The Promyelocytic Leukemia Zinc Finger Protein as a Novel Regulator of Dopamine D_1R Class

Josephine Zein

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Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

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Abstract

Compromised D_1R-class signaling is implicated in several neurodegenerative disorders such as in the case of L-DOPA-induced dyskinesia (LID) seen in individuals suffering from Parkinson's disease (PD). Therefore, extensive research is required to be able to therapeutically target the altered mechanisms in D_1R-class responsiveness which develop in these psychomotor disorders. One main component that is yet to be fully delineated is the role of proteins that interact with the intracellular loops of the D_1R-class. Thus, to gain further insight into the protein-protein interaction networks implicated in the regulation of the D_1R-class, our laboratory performed yeast two-hybrid screening to characterize the interactions between PLZF and the cytoplasmic tail of the D_1R, as well as PLZF and the third intracellular loop of the D_5R. Furthermore, our lab identified endogenous PLZF-D_5R complex formation within the rat hippocampus and striatum. Interestingly, PLZF is described as a multifunctional transcription regulator and has been characterized as a cytosolic and nuclear protein expressed in developing and mature brains. PLZF has also been shown to interact with the agonist-stimulated GPCR AT_2R, internalize with AT_2R, shuttle to the nucleus upon prolonged AngII exposure and bind to the constitutively activated Gαo subunit. Based on these findings, I hypothesized that PLZF regulates D_1R-class function through complex formation, signaling and trafficking mechanisms. Herein, I demonstrate using co-transfected HEK293 cells that upon agonist exposure, there is an increase in the association between PLZF and the D_1R-class. The PLZF-D_1R and PLZF-D_5R interactions do not alter the ligand binding affinities of the receptors. Interestingly, the co-expression of β-arrestin 2 with PLZF and either the D_1R or D_5R results in decreased recruitment of β-arrestin 2 to the receptors. Furthermore, the PLZF-D_1R interaction results in increased

cAMP and ERK activation, while PLZF-D_5R interaction results in decreased cAMP and ERK activation. PLZF also increases the cellular surface and total expression of the D_1R but does not have any effect on the D_5R surface and total expression. The distinct contrasting effect of PLZF interaction with the D_1R-class is also observed in the disrupted internalization of the D_1R vs. the facilitated internalization of the D_5R. Moreover, β-arrestin 2 co-expression with PLZF impedes the extent of internalization for both the D_1R and D_5R. Finally, PLZF distinctly regulates D_1R-class recycling by possibly accelerating D_1R recovery and delaying D_5R recovery. These novel findings may provide a better understanding of the molecular mechanisms involved in the etiology of neuropsychiatric diseases and potentially facilitate the development of treatments for dopamine-related disorders.

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List of Abbreviations

AA: Ascorbic acid

AC: Adenylyl cyclase

ADHD: Attention deficit hyperactivity disorder

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AngII: Angiotensin II

AP-2: Adaptor protein-2

AP: Action potential

APL: Acute promyelocytic leukemia

AT_1R: Angiotensin II type 1 receptor

AT_2R: Angiotensin II type 2 receptor

ATP: Adenosine triphosphate

BCS: Bovine calf serum

B_{max}: Maximal binding capacity

BSA: Bovine serum albumin

BTB: Bric à brac, tramtrack and broad

BTBD6: BTB domain containing 6

Ca^{2+}: Calcium

CAMKII: Calcium/calmodulin-dependent kinase II

cAMP: Cyclic adenosine monophosphate

CB: Cannabinoid

CDK: Cyclin-dependent kinase

CK: Casein kinase

CNS: Central nervous system

Co-IP: Co-immunoprecipitation

COMT: Catechol-O-methyltransferase

COP: Coatomer protein complex

CREB: cAMP response element-binding protein

CT: Carboxyl-terminus

Cul-3: Cullin-3

Cys: Cysteine

D_1R: Dopamine D_1 receptor

D_2R: Dopamine D_2 receptor

D_3R: Dopamine D_3 receptor

D_4R: Dopamine D_4 receptor

D_5R: Dopamine D_5 receptor

DA: Dopamine

DAG: Diacylglycerol

DAR: Dopamine Receptor

DARPP-32: DA and cAMP-regulated phosphoprotein 32

DAT: Dopamine transporter

DHX: Dihydrexidine

DMEM: Dulbecco's modified eagle medium

DOPA decarboxylase: L-amino acid decarboxylase

DRIP: Dopamine receptor interacting protein

EC_{50}: Half maximal effective concentration

EDTA: Ethylenediaminetetraacetic acid

EL: Extracellular loop

E_{max}: Maximal efficacy

EMEM: Eagle's minimal essential medium

ENTH: Epsin NH_2 terminal homology

ER: Endoplasmic reticulum

ERK: Extracellular signal regulated kinase

FBS: Fetal bovine serum

FGF: Fibroblast growth factors

G-protein: Guanine nucleotide-binding protein

$GABA_A$R: γ-aminobutyric acid type A receptor

GDNF: Glial cell-derived neurotrophic factor

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factor

GPCR: G protein-coupled receptor

GRK: GPCR kinase

GSK3ß: Glycogen synthase kinase 3 beta

GTP: Guanosine triphosphate

HB-EGF: Heparin-binding epidermal-like growth factor

HD: Huntington's disease

HDAC: Histone deacetylase

HEK293: Human embryonic kidney 293

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His: Histidine

HRP: Horseradish peroxidase

HSC: Hematopoietic stem cells

IB: Immunoblot

IFN: Interferon

IL: Intracellular loop

IP_3: Inositol triphosphate

L-DOPA: L-3,4-dihydroxyphenylalnine

LID: Levodopa-induced dyskinesia

MAO: Monoamine oxidase

MAPK: Mitogen activated protein kinase

MDD: Major depressive disorder

mGluR5: Metabotropic glutamate receptor 5

MSNs: Medium spiny neurons

mTORC: Mammalian TOR complex

N-CoR: nuclear corepressors

NAc: Nucleus accumbens

NF-M: Neurofilament-M

NMDAR: N-methyl-D-aspartate receptor

NPC: Neural progenitor cells

NPY: Neuropeptide Y

NSC: Neural stem cells

NSF: N-ethylmaleimide-sensitive factor

NT: Amino-terminus

OD: Optical density

OPD: O-Phenylenediamine Dihydrochloride

PBS: phosphate-buffered saline

PD: Parkinson's disease

PDZ: PSD-95-Disc large Zonula occludens-1

PFC: Prefrontal cortex

PIP2: Phosphatidylinositol biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PLL: Poly-L-lysine

PLZF: Promyelocytic leukemia zinc finger

PMSF: Phenylmethylsulphonyl fluoride

PNS: Peripheral nervous system

POZ: Poxvirus and zinc finger

PP1: Protein phosphatase 1

PSD-95: Post synaptic density 95

RA: Retinoic acid

RAMP: Receptor Activity-Modifying Protein

RARα: Retinoic acid receptor alpha

RAS: Renin-angiotensin system

RD2: Repression domain 2

Redd1: Regulated in development and DNA damage responses 1

RER: Renin/prorenin receptor

RGS: Regulators of G-protein signaling

ROC1: Regulator of cullins-1

SNpc: Substantia nigra pars compacts

SNX-1: Sorting nexin-1

SPC: Spermatogonial progenitor cells

SSC: Spermatogonial stem cells

STEP: Striatal-enriched tyrosine phosphatase

SUMO: Small ubiquitin-like modifier

TBS-T: Tris-buffered saline containing Tween 20

TH: Tyrosine hydroxylase

THX: Thiothixene

TM: Transmembrane

ULK1: Unc-51 like autophagy activating kinase 1

VMAT: Vesicular monoamine transporters

VTA: Ventral tegmental area

Zbtb16: Zinc finger and bric à brac, tramtrack and broad BTB domain containing 16

ZNF145: Zinc finger protein 145

β1-AR: β1-adrenergic receptor

β2-AR: β2-adrenergic receptor

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INTRODUCTION

1. Overview of the Dopaminergic System

1.1. Dopamine Synthesis and Function

Dopamine (DA), also known as 3,4-dihydroxyphenylethylamine, is a metabolite of the amino acid tyrosine and the main catecholaminergic neurotransmitter in the mammalian brain. DA synthesis starts in the presynaptic neurons and is synthesized in several steps **(Fig. 1)**. The DA precursor L-tyrosine is hydroxylated to 3,4-dihydroxy-L-phenylalanine (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH). Next, L-DOPA is decarboxylated by the aromatic L-amino acid decarboxylase (DOPA decarboxylase) to DA. DA is then stored by vesicular monoamine transporters (VMAT) into storage vesicles and excess DA in the presynaptic terminal is degraded by monoamine oxidase (MAO). An action potential (AP) occurs when the neuron is depolarized, leading to an influx of Ca^{2+} and the release of DA in the synaptic cleft. The excess DA in the postsynaptic terminal is metabolized by the catechol-O-methyltransferase (COMT). Lastly, the dopamine transporter (DAT) drives the reuptake of extracellular DA into the presynaptic cleft, controlling the spatial and temporal dynamics of DA neurotransmission (Daubner et al., 2011; Verheij & Cools, 2008).

DA plays a critical role in the regulation of a variety of central nervous system (CNS) functions including locomotor activity, cognition, positive reinforcement, food intake, sleep, learning, and memory. In the peripheral nervous system (PNS), DA also controls vital physiological functions such as olfaction, retinal processes, hormonal regulation as well as gastrointestinal, cardiovascular, immune, renal and sympathetic system regulation. Since DA regulates various critical physiological functions, it is evident that dopaminergic dysfunctions contribute to the pathophysiology of multiple human disorders, including Parkinson's disease

(PD), Levodopa-induced dyskinesia (LID), and Huntington's disease (HD). Furthermore, DA dysregulation also contributes to the development of a host of neuropsychiatric conditions such as schizophrenia, major depressive disorder (MDD), attention deficit hyperactivity disorder (ADHD) and bipolar disorder (Beaulieu, Espinoza, & Gainetdinov, 2015; Beaulieu & Gainetdinov, 2011; Missale et al., 1998).

1.2. Major Dopaminergic Pathways

DA neurons are mainly localized in three brain regions; the ventral tegmental area (VTA) of the midbrain, the substantia nigra pars compacta (SNpc), the arcuate and the periventricular nuclei of the hypothalamus. The axons of these neurons project to different regions of the brain through four major dopaminergic pathways, known as mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular pathways **(Fig. 2)**. First, DA neurons in the VTA project to the nucleus accumbens (NAc) via the mesolimbic pathway. Second, the dopaminergic projections within the mesolimbic pathway originate in the VTA and travel to the prefrontal cortex (PFC). Third, DA neurons in the SNpc project to the dorsal striatum via the nigrostriatal pathway. The final pathway is the tuberoinfundibular pathway. In this pathway, DA neurons begin in the arcuate and periventricular nuclei of the hypothalamus and project to the median eminence to release DA into the pituitary gland (Beaulieu & Gainetdinov, 2011; Klein et al., 2019; Missale et al., 1998).

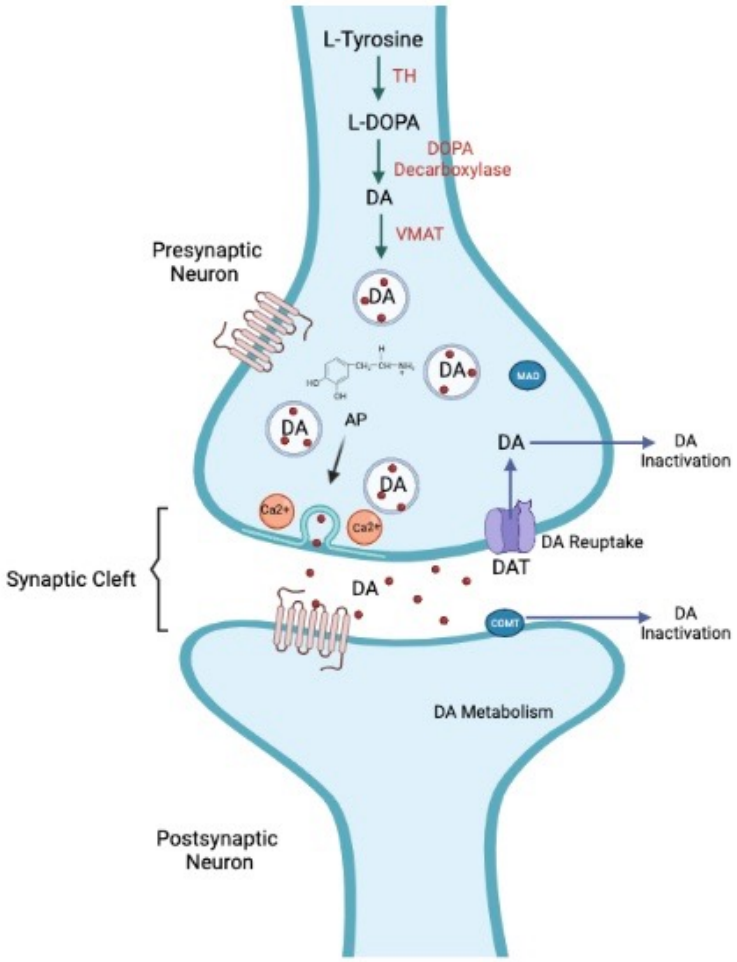


Figure 1. Schematic Diagram of Dopamine Synthesis
In the presynaptic neuron, DA is synthesized from L-tyrosine. Tyrosine hydroxylase (TH) is the rate limiting-enzyme that converts L-tyrosine to L-DOPA. Decarboxylation of L-DOPA by DOPA decarboxylase produces DA. Newly synthesized DA is then packaged into storage vesicles by VMAT2. Action potential (AP) activity results in the increase of Ca^{2+} influx in the synaptic terminal which allows the DA-storing vesicles to fuse with the plasma membrane and release DA into the synaptic cleft. Once DA is released in the synaptic cleft, DA binds to dopaminergic autoreceptors located presynaptically or activates postsynaptic DA receptors leading to the activation of different signaling pathways. Remaining DA in the postsynaptic cleft is then degraded by COMT and DA reuptake by DAT terminates DA activity. COMT is also expressed on glial cells (not shown). Created with BioRender.com

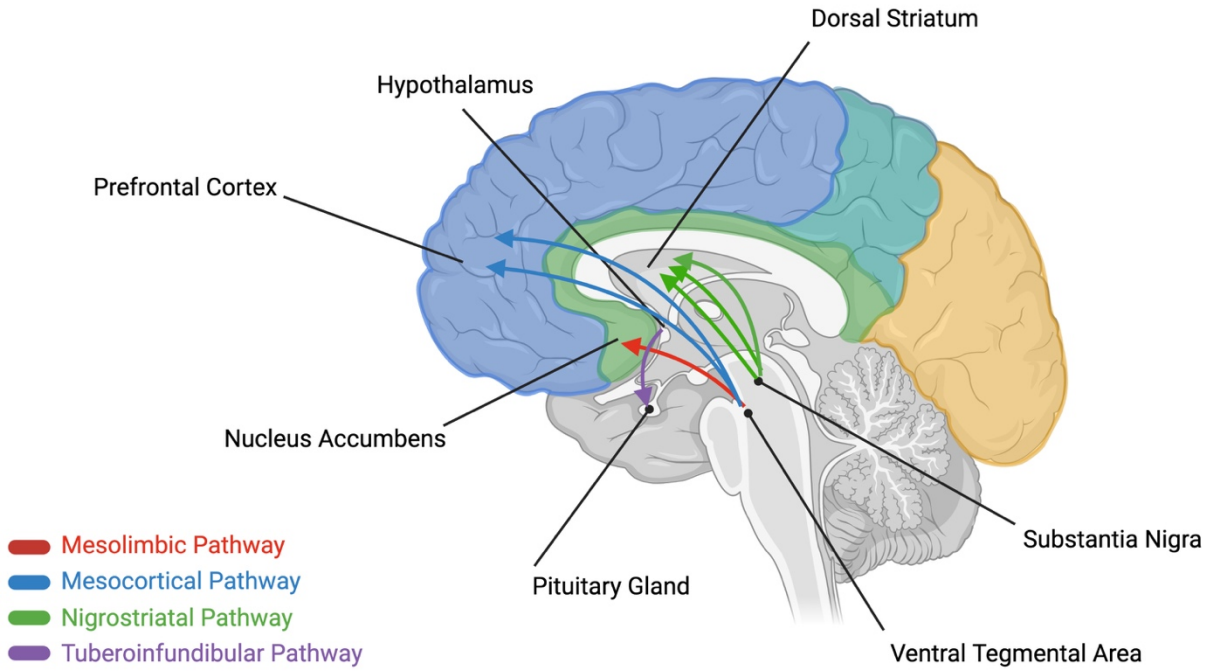


Figure 2. The Major Dopaminergic Neuronal Pathways
There are four major dopaminergic pathways. 1. The mesolimbic pathway projects from the ventral tegmental area to the nucleus accumbens and other limbic areas (e.g. hippocampus and amygdala, not shown). 2. The mesocortical pathway: projects from the ventral tegmental area to the prefrontal cortex and sensorimotor cortex (not shown). 3. The nigrostriatal pathway projects from the substantia nigra to the dorsal striatum. 4. The tuberoinfundibular pathway projects from the hypothalamus to the pituitary gland. Created with BioRender.com

2. G-Protein Coupled Receptors (GPCRs)

Once DA is synthesized in both the CNS and the periphery, it exerts its actions upon binding to G protein-coupled receptors (GPCRs). GPCRs comprise the largest family of cell surface receptors that mediate numerous physiological functions, including smell, taste, vision, immune response, neurological and cardiovascular regulation (Kim & Chung, 2020). GPCRs are versatile, seven-transmembrane (7TM) domain proteins that regulate an array of intracellular signaling cascades in response to external stimuli, such as ions, odorants, hormones, neurotransmitters and other stimuli (Hilger et al., 2018). Therefore, GPCRs represent attractive drug targets as they play a crucial role in physiology and disease.

2.1. GPCR Structure

All GPCRs share a similar structure consisting an extracellular amino-terminus (NT), followed by seven hydrophobic transmembrane α-helices (TM1-TM7) which are linked by three alternating extracellular loops (EL1-EL3) and intracellular loops (IL1-IL3), and finally an intracellular carboxyl-terminus (CT). The transmembrane domains present the highest degree of sequence conservation, while the intracellular and extracellular domains exhibit great variability in size and amino acid sequence. The NT contains N-glycosylation sites, which allow for cell surface expression. In addition, there are two cysteine residues that form a disulfide bridge between the EL1 and EL2 for normal protein folding, as well as a cysteine residues in the CT domain which serve as a site for palmitoylation. This lipid modification forms a putative fourth intracellular loop (IL4) which has been characterized in crystallized GPCRs as an alpha-helix region referred to as helix 8. Evidently, the 7TM domains are known to manage ligand binding whereas the CT and ILs interact with heterotrimeric guanine nucleotide-binding protein (G-

proteins) and various intracellular components (Fredriksson et al., 2003; Hoffmann et al., 2008; Luttrell, 2008) **(Fig. 3)**.

2.2. GPCR-mediated Activation of G-proteins

The classical signal transduction through GPCRs is dependent on the receptor-mediated activation of heterotrimeric G proteins, which connect receptors to downstream effectors to produce diverse cellular responses. The heterotrimeric G-proteins are composed of three subunits, known as Gα, Gβ and Gγ. Four major G-protein families (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) have been classified based on primary sequence homology between the 21 identified human Gα isotypes encoded by 16 distinct genes. Members of the Gα family range in size from 39-52 kDa and all Gα structures are composed of two domains: a GTPase domain and a helical domain (Downes & Gautam, 1999; Oldham & Hamm, 2006; Simon et al., 1991). The helical domain is composed of six α-helices that cover the nucleotide binding site (guanosine diphosphate (GDP)/guanosine triphosphate-binding site (GTP)-binding site), concealing the bound nucleotides in the core of the protein (Warner et al., 1998). Since the helical domain is the most divergent among Gα subunits, it may play an important role in coupling specific G proteins to effectors (Liu et al., 1998). The GTPase domain is known to hydrolyze GTP and contains sites for the Gβγ dimer. The Gβ subunit has 6 different isoforms and a size of approximately 35-39 kDa, and forms a non-dissociable functional heterodimer with Gγ subunit, which has 12 different isoforms and is 7-8 kDa in size (Oldham & Hamm, 2006).

In the inactive state, the Gα subunit is bound to the Gβγ subunits to form the heterotrimeric G-protein complex. Upon ligand binding, the GPCR shifts to an active state and undergoes conformational changes in the intracellular domains of the receptor. These changes

lead to the association of the receptor with a variety of heterotrimeric G-proteins, which prompts the exchange of GDP for GTP, resulting in the dissociation of the Gα subunit from the Gβγ complex. Both the GTP-bound Gα subunit and the released Gβγ complex are then able to interact with intracellular or membrane effectors, such as ion channels or enzymes. To restore the initial inactive conformation as well as its affinity for the Gβγ complex, the intrinsic GTPase activity of the Gα subunit hydrolyzes GTP into GDP. This process requires the interaction of Gα with proteins called regulators of G protein signaling (RGS), which catalyze the intrinsic GTPase activity. Without this interaction, the hydrolysis of GTP is slow **(Fig. 4)**.

2.3. Classification of GPCRs

The human genome contains approximately 800 GPCRs, rendering it the largest family of membrane proteins. GPCRs are categorized into five classes on the basis of function and sequence similarities, namely rhodopsin-like receptors (R, 701), secretin family (S, 15), metabotropic glutamate receptors (G, 15), adhesion receptors (A, 24) and frizzled/taste2 receptors (F, 24) (Fredriksson et al., 2003; Lee et al., 2017). The most studied group of GPCRs is the rhodopsin family as it contains the largest number of receptors and accounts for 80% of receptors in humans. The rhodopsin family is further subdivided into four main groups: α (biogenic amine receptors including dopamine receptors (DARs), serotonin receptors, prostaglandin receptors, opsins, melatonin receptors, as well as melanocortin and cannabinoid (MECA) receptors, β (neurotensin and neuropeptide Y (NPY) receptors) , γ (somatostatin and chemokine receptors), δ (MAS-oncogene-related receptors, glycoprotein receptors, purine receptors, and the olfactory receptors) (Fredriksson et al., 2003).

The rhodopsin family of receptors share a common structural feature of approximately 20 highly conserved amino acids in the cytoplasmic side of the TM domains. These residues have been shown to be critical for proper protein folding and receptor stability upon receptor activation (Moreira, 2014). Specifically, members of the rhodopsin family share a conserved aspartate-arginine-tyrosine (DRY) motif located at the border between the TM3 and IL3. This motif is important for protein stabilization, specificity, and maintaining receptor inactivity (Moreira, 2014; Rovati et al., 2007). In addition, it is known that the intracellular loops of the rhodopsin receptors vary in length and amino acids, yet the TM domains are highly conserved. Specifically, the IL2 is the most conserved domain with 20 ± 2 amino acids while the IL3 is the most divergent domain with 41 ± 43 amino acids. The NT and CT domains contain approximately 62 ± 98 and 53 ± 26 amino acids, respectively (Karnik et al., 2003; Moreira, 2014).

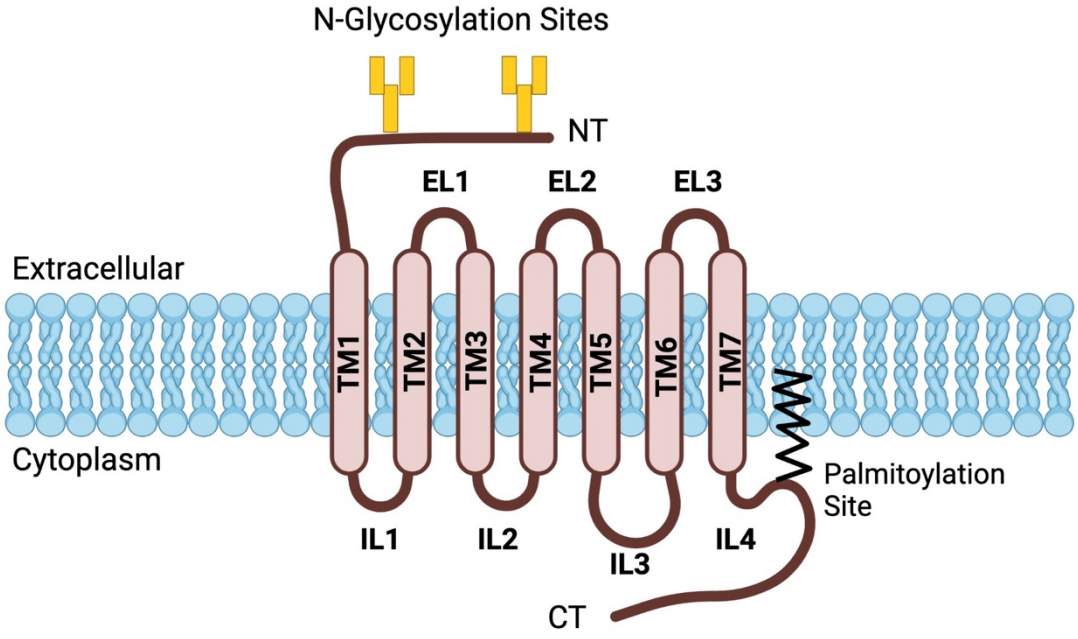


Figure 3. Schematic Diagram of General GPCR Structure
GPCR structure contains seven alpha-helix TM domains, connected by three ELs and three ILs. The external segment of the receptor is the NT and the internal segment is the CT. The TM domains form the ligand binding site while the three ILs and CT are sites that allow interaction with the G-protein. The NT of the receptor contains N-glycosylation sites while the CT contains cysteine residues serving as palmitoylation sites forming the IL4. Created with BioRender.com

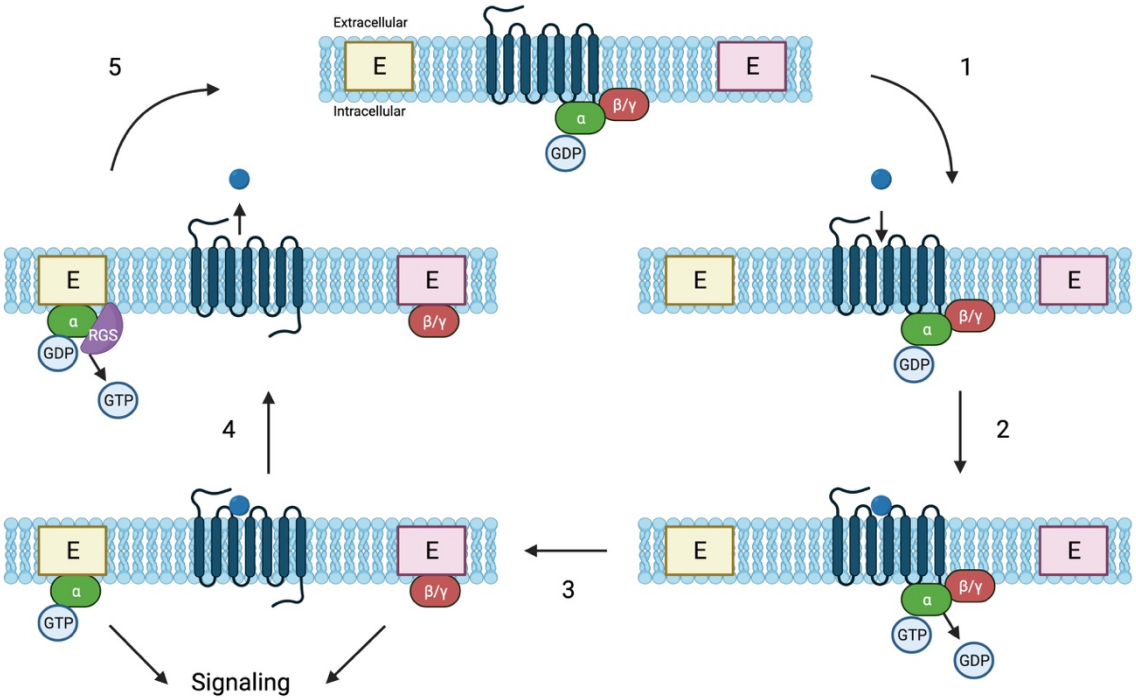


Figure 4. Schematic Diagram of Heterotrimeric G protein Activation
In the inactive state of the GPCR, the heterotrimeric G-protein complex consists of GDP-bound Gα associated with Gβγ subunits. 1: Ligand binding to GPCRs produces conformational changes in the intracellular domains of the receptors and the GPCR is now in an active state. 2: Changes in the intracellular domains trigger the exchange of GDP for GTP. 3: The Gα subunit dissociates from the Gβγ complex, and both Gα and Gβγ can interact with different effector partners leading to downstream signaling. 4: The intrinsic GTPase activity of Gα-subunit will then hydrolyze the GTP into GDP which requires interaction between Gα-subunit and RGS. 5: G protein subunits reunite again to form a non-functional heterotrimeric Gαβγ complex and to return back to the inactive resting state. Created with BioRender.com

2.4. Canonical GPCR Signaling Pathways

The biophysical and crystallographic analysis of GPCRs has validated the functionalities and properties of various structural elements shared by the activation mechanisms of this family of receptors. For instance, it has been shown that GPCR activation through ligand binding causes an outward rotation of the intracellular end of TM6, which opens a crevice within the intracellular surface of the receptors into which a G protein can bind (Moreira, 2014; Oldham & Hamm, 2008).

The classical signaling pathway of GPCRs begins with the activation of the $G\alpha_s$ which stimulates adenylyl cyclase (AC), while activation of $G\alpha_i$ inhibits AC. AC catalyzes the conversion of adenosine triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP), which then activates cAMP-dependent protein kinase A (PKA). In contrast, the activation of $G\alpha_q$ results in the stimulation of phospholipase-C (PLC), which catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 increases the release of calcium (Ca^{2+}) through activation of IP_3 receptors located on the endoplasmic reticulum (ER), which leads to the activation of protein kinase C (PKC) (Seyedabadi et al., 2019; Wettschureck & Offermanns, 2005).

2.5. Regulation of GPCR Signaling

Studies have shown that GPCRs interact with at least three families of proteins in an agonist-dependent manner, including the heterotrimeric G proteins, GPCR kinases (GRKs) and arrestins (Jean-Charles et al., 2017; Weis & Kobilka, 2018).

2.5.1. Negative Regulation of GPCRs

Receptor responsiveness is regulated through various mechanisms that dampen GPCR signaling. The GPCR is mainly the target for extensive negative regulation and the processes that regulate GPCR responsiveness at the receptor level are usually categorized based on mechanism, into receptor desensitization (homologous and heterologous desensitization), internalization, recycling and downregulation. This ultimately leads to a sequence of steps including the uncoupling of the receptor from the G protein, removal of receptors from the plasma membrane, receptor recycling or degradation, and reduced synthesis of new receptors (Luttrell, 2008).

2.5.1.1. Heterologous Desensitization

Within seconds of agonist exposure, the receptor becomes phosphorylated and desensitization is initiated. The serine and threonine residues within the cytoplasmic loops and CT domains are phosphorylated by second messenger-dependent protein kinases, including PKA and PKC (Luttrell, 2008). Phosphorylation of these sites directly impedes receptor-G protein coupling. Most importantly, agonist occupancy of the target GPCR is not required for this process (Freedman & Lefkowitz, 1996). Therefore, this mechanism is called heterologous desensitization because the receptors that do not have a bound agonist, including receptors for other ligands, can be desensitized by the activation of second messenger-dependent protein kinases (Freedman & Lefkowitz, 1996; Luttrell, 2008).

2.5.1.2. Homologous Desensitization

Homologous desensitization is a two step-process which involves receptor phosphorylation by specific GRKs followed by the binding of an arrestin protein, whose role is

to physically uncouple the receptor and G protein. Specifically, GRKs phosphorylate agonist-bound receptors on serine and threonine residues present in their IL3 and CT domains. This produces high-affinity for β-arrestin binding and results in steric hindrance of G protein coupling. There are seven known GRKs. GRK1 and GRK7 are retinal kinases, whereas GRK2-GRK6 are more widely expressed (Stoffel et al., 1997). Unlike PKA and PKC, GRKs preferentially phosphorylate receptors that are in the agonist-occupied conformation. Moreover, GRK phosphorylation alone has minimal direct effect on receptor-G protein coupling and receptor desensitization. Alternatively, the main function of GRKs in GPCR desensitization is to increase receptor affinity for arrestins. Therefore, it is the binding of arrestin to receptor domains involved in G protein coupling that leads to homologous desensitization **(Fig. 5)**.

2.5.2. β-arrestin

There are two isoforms of β-arrestins. β-arrestin 1 and β-arrestin 2 are expressed in most tissues and play an important role in regulating signal transduction of various GPCRs (Reiter & Lefkowitz, 2006). Studies of the β2-adrenergic receptor (β2-AR) reveal that receptor activation causes the translocation of β-arrestins from the cytoplasm to the cell membrane and the interaction of β-arrestins with the activated GPCR (Claing et al., 2002). This interaction results in the internalization of the receptor and attenuation of signaling by essentially uncoupling the receptor from its cognate G proteins (Claing et al., 2002; Ma & Pei, 2007). Studies have shown that GPCRs can signal through coupling to non G-protein effectors like β-arrestins (Perry & Lefkowitz, 2002).

β-arrestins are known to behave as signal terminators or signal transducers, as well as scaffolding proteins which allow interactions with several intracellular signaling molecules

(Luttrell, 2008). As a result, β-arrestins connect GPCRs to various signaling pathways such as the mitogen activated protein kinase (MAPK) cascades and proto-oncogene tyrosine kinase Src family, mediating G protein independent signaling (Luttrell, 2008; Ma & Pei, 2007). For instance, the role of β-arrestins in the regulation of MAPK cascades is an extensively studied signaling mechanism. G-protein-dependent extracellular signal regulated kinase (ERK) activation appears to be fast and transient and can be interrupted by pertussis toxin or PKA inhibitors (Gesty-Palmer et al., 2006). Whereas, β-arrestin-dependent ERK activation is slower in onset and durable, and it is sensitive to depletion of β-arrestins but not G_i/PKA inhibition or loss of G_s (Shenoy et al., 2006).

In addition, activation of ERKs does not consistently induce their nuclear translocation and transcriptional responses, as G protein-dependent ERK activation does not result in the translocation of activated ERKs from the cytoplasm to the nucleus (Lefkowitz & Shenoy, 2005). Specifically, the ERK1/2 activated by the β-arrestin scaffold is mostly confined to cytoplasmic locations such as endocytic vesicles which results in the attenuation of the ERK effect on phosphorylation of substrates in the nucleus (Jafri et al., 2006). Instead, retention of ERKs in the cytoplasm affects the cellular response through phosphorylation and activation of transcription factors in the cytoplasm and their translocation to the nucleus (Ma & Pei, 2007).

The rhodopsin subfamily of GPCRs are known to have higher affinity for binding to β-arrestin 2 than β-arrestin 1 (Kohout et al., 2001). In addition to negative regulation of GPCR-mediated signaling, β-arrestins are also known to play a role in receptor trafficking and signaling (Lefkowitz & Shenoy, 2005). The CT of β-arrestin contains a binding motif for clathrin and β2-adaptin subunit of the adaptor protein-2 (AP-2) (Laporte et al., 1999). This allows β-arrestin to target GPCRs to clathrin-coated pits for endocytosis and removal from the plasma membrane

(Luttrell, 2008). The GPCRs are then ushered to endosomes for sorting either to the lysosomes for degradation or dephosphorylation, release of arrestin, resensitization and then recycling back to the cell surface (Kohout et al., 2001; Luttrell, 2008) **(Fig. 5)**.

2.5.3. Positive Regulation of GPCRs

The interactions between GPCRs and various other proteins positively modulate GPCR signaling, ligand binding, coupling to G protein and effectors, and targeting specific subcellular locations. Studies suggest that GPCRs are organized into multi-receptor "signalosomes" that impact the receptor responsiveness (Luttrell, 2008). These protein-protein interactions include GPCR homodimerization (two identical receptors interacting with each other) and heterodimerization (two distinct receptors interacting with each other). Various GPCR dimers form during synthesis, and in some cases, such as $GABA_B$ receptors, dimerization is necessary for trafficking nascent receptors to the plasma membrane (Kniazeff et al., 2002; Luttrell, 2008).

Additionally, positive regulation of GPCRs is achieved through the interaction with Receptor Activity-Modifying Proteins (RAMPs), and the binding of scaffolding proteins to the GPCR IL3 and CT (Luttrell, 2008). The three known RAMP proteins are 148-174 amino acid single membrane-spanning glycoproteins with large extracellular domains and short cytoplasmic domains (Sexton et al., 2001). In addition, the post synaptic density of 95 kDa (PSD95)-Disc large-Zonula occludens-1 (PDZ) domains are protein-protein recognition domains that allow binding to short peptide motifs located in the CT of proteins, which serve as scaffolds for protein trafficking, localization and complex assembly (Bockaert et al., 2003; Brady & Limbird, 2002; Luttrell, 2008). Therefore, these GPCR interacting proteins are able to modulate the function, trafficking, and ligand specificity of the receptors (Luttrell, 2008).

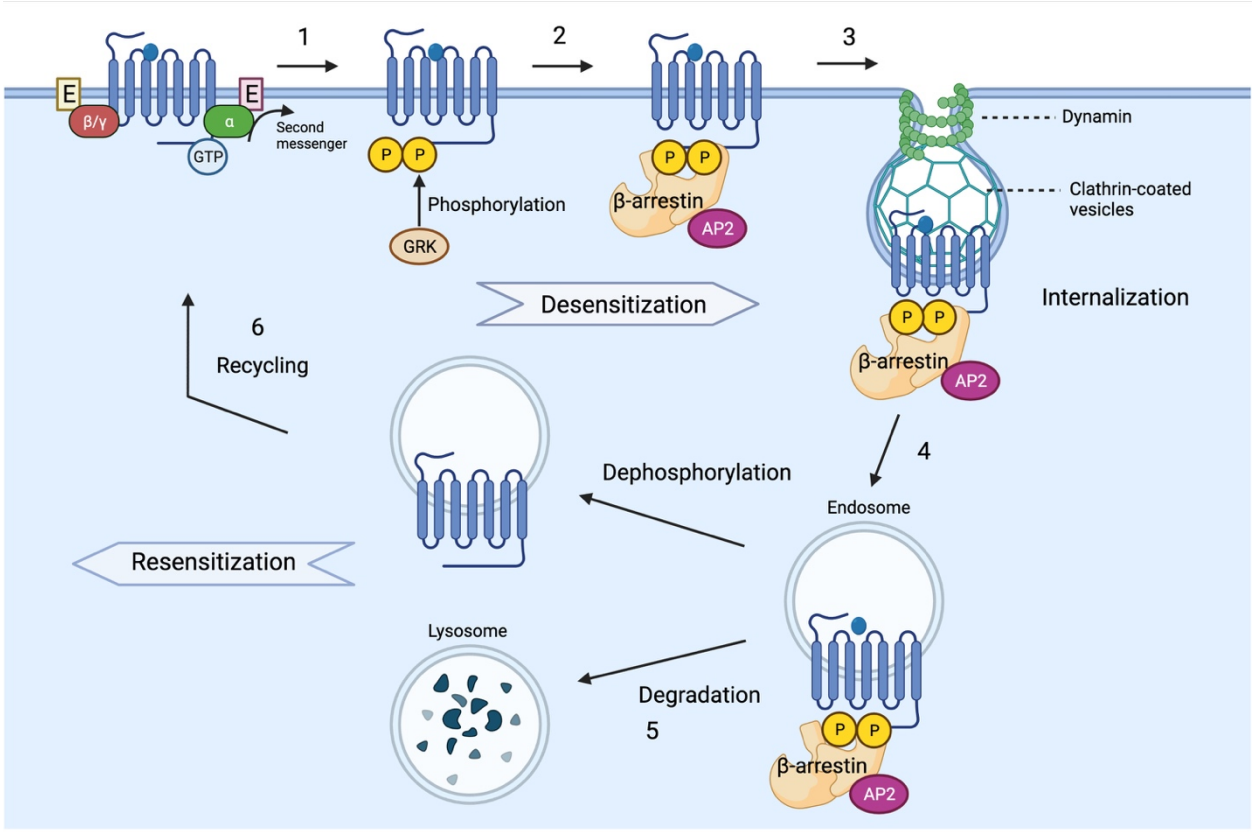


Figure 5. Schematic Diagram of GPCR Desensitization and Trafficking
1: Exposure to ligand leads to rapid uncoupling of the GPCR from its G protein due to receptor phosphorylation by GRKs on the CT and ILs. **2**: Phosphorylation of activated GPCRs by GRKs promotes the binding of β-arrestin to phosphorylated receptors, which disrupts the GPCR/G protein interaction leading to desensitization. **3**: Following receptor phosphorylation, GPCR-β-arrestin complex is targeted for endocytosis and dynamin-dependent internalization of GPCRs occurs through clathrin-coated vesicles. **4**: Internalized GPCRs are sorted into **5**: lysosomes for degradation or **6**: dephosphorylated and recycled back to the cell surface. Created with BioRender.com

3. Family of Dopamine Receptors (DARs)

3.1. Classification of DARs

The diverse physiological functions controlled by DA in the central and peripheral nervous systems are mediated by the five subtypes of DARs (D_1R, D_2R, D_3R, D_4R and D_5R). DARs belong to the GPCR superfamily and are classified into two major groups: the D_1R-class (D_1R and D_5R), and D_2R-class (D_2R, D_3R and D_4R), based on their canonical signaling pathways, structure, and pharmacological properties. The D_1R-class stimulate AC through coupling to the stimulatory or olfactory heterotrimeric G α protein ($G\alpha_{s/olf}$) and thereby stimulating the production of the second messenger cAMP. In contrast, the D_2R-class inhibit AC by coupling to the inhibitory/o heterotrimeric G α protein ($G\alpha_{i/o}$) and thus inhibit the production of cAMP (Beaulieu et al., 2015; Beaulieu & Gainetdinov, 2011; Missale et al., 1998).

The D_1R-class primarily acts through the $G\alpha_{s/olf}$-cAMP-PKA-DA and cAMP-regulated phosphoprotein 32 kDa (DARPP-32)- dependent pathways. On the other hand, the D_2R-class acts via both the $G\alpha_{i/o}$-cAMP-PKA-DARPP-32 pathway and through the G protein βγ subunit complex (Gβγ)-mediated signaling pathways. The D_1R-class canonical signaling pathway ultimately leads to an increase in neuronal excitability by reducing the activity of γ-aminobutyric acid type A receptors ($GABA_AR$), K^+ channels, N- and P/Q-type Ca^{2+} channels, and Na^+ channels, and augmenting the activity of L-type Ca^{2+} channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDAR). Conversely, the D_2R-class canonical signaling pathway serves to reduce neuronal excitability through enhancing K^+ channel activity and reducing the activity of N- and P/Q-type Ca^{2+}

channels, Na^+ channels, L-type Ca^{2+} channels, $GABA_AR$, AMPAR, and NMDAR (Beaulieu et al., 2015; Beaulieu & Gainetdinov, 2011; Missale et al., 1998).

The classification of DARs is also based on the genetic structure and the absence or presence of introns in the coding sequence of the D_1R- and D_2R-classes. The D_1R-class genes do not contain introns, therefore D_1R and D_5R exist as a single isoform. In contrast, the genes encoding the D_2R-class contain introns, however only the D_2R contains more than one functional splice variant, called the short (D_{2S})R and long (D_{2L})R isoforms which are generated by alternative splicing of a 87-base pair exon between the fourth and fifth intron. The (D_{2L})R differs from (D_{2S})R by the addition of 29 amino acids in the IL3 (Beaulieu & Gainetdinov, 2011; Missale et al., 1998) **(Fig. 7)**. Structural analysis of DARs reveals the shared homology between the subtypes belonging to the D_1R- and D_2R-class. The D_1R and D_5R share a 80% identity in their TM domains **(Fig. 6)**, while the D_2R and D_3R share a 75% identity and the D_2R and D_4R share a 53% identity, in their TM domains (Beaulieu & Gainetdinov, 2011; Civelli et al., 2003; Missale et al., 1998) **(Fig. 7)**. The differences between the D_1R- and D_2R-classes within the 7TM regions results in a considerably higher DA affinity for D_2R-class in comparison to D_1R-class. The DARs are also grouped into the D_1R- and D_2R-class due to the major structural differences present within the intracellular CT and the IL3 regions. Specifically, the D_1R-class have a CT approximately seven times longer than that of the D_2R-class, while in contrast the D_2R-class have a relatively longer IL3 in comparison to the D_1R-class (Beaulieu & Gainetdinov, 2011; Missale et al., 1998) **(Fig. 6, Fig. 7)**.

Additionally, the D_1R- and D_2R-classes vary in their post-translational modifications. All DARs display N-glycosylation sites in the extracellular NH_2-terminal. Specifically the D_1R-class possess two N-glycosylation sites, one in the NH_2-terminal and the other in the EL2. The D_2R

has four potential glycosylation sites, the D_3R possesses three and the D_4R has only one (Beaulieu & Gainetdinov, 2011; Gingrich & Caron, 2003; Missale et al., 1998). Moreover, the CT in the D_1R-class is rich in serine and threonine residues while the IL3 in the D_2R-class is rich in serine and threonine residues. Both classes of receptors contain a cysteine residue that may become palmitoylated perhaps to anchor the CT to the membrane. In the D_1R-class, this cysteine residue is localized near the beginning of the CT and in the D_2R, it is situated near the end of the CT (Beaulieu & Gainetdinov, 2011; Jackson & Westlind-Danielsson, 1994; Missale et al., 1998).

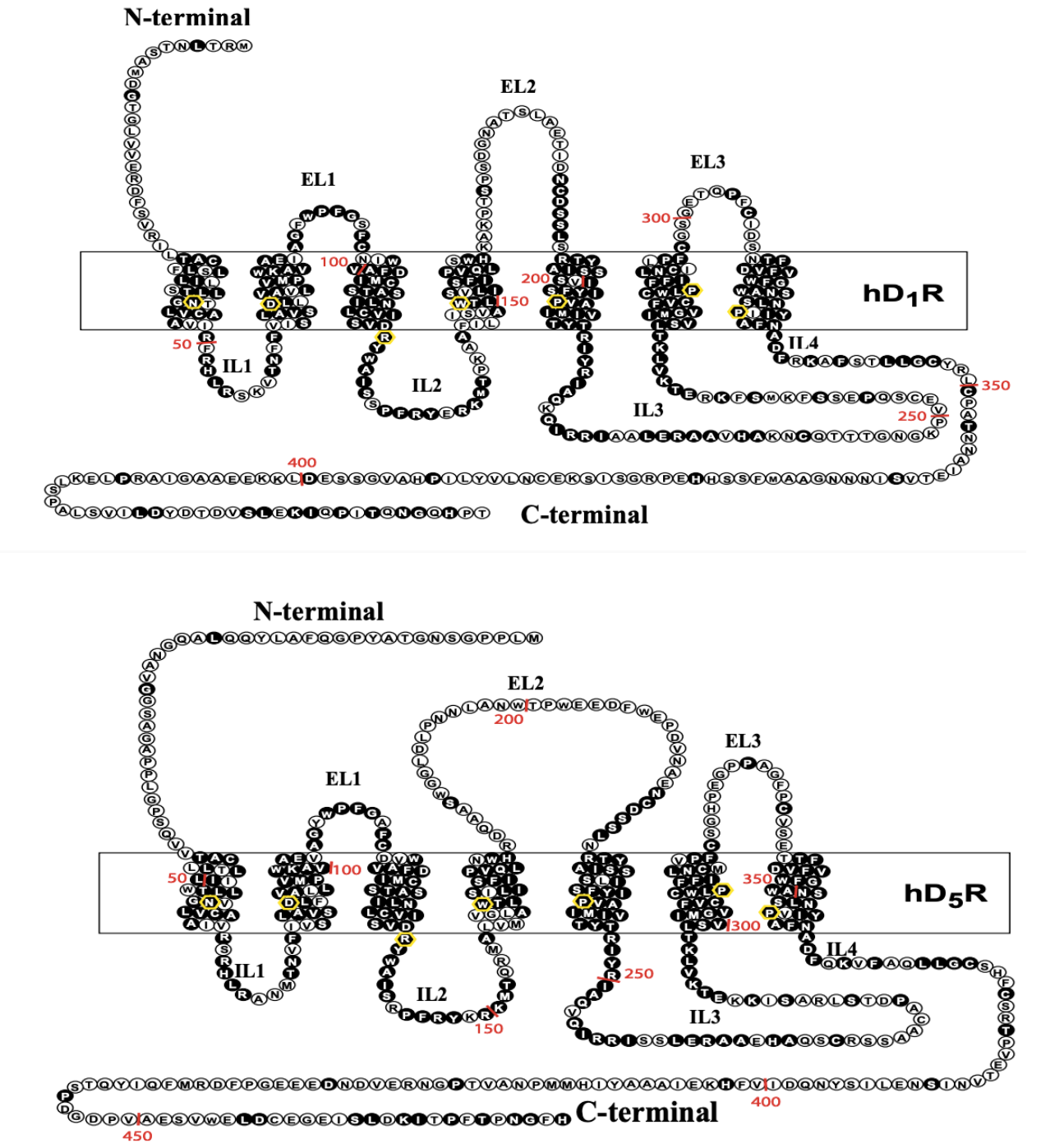


Figure 6. Secondary Structures and Amino Acid Sequences of the Human D_1R and D_5R
Black circles represent identical amino acid residues conserved between D_1R and D_5R and open circles represent the divergent residues between the two subtypes. Taken from (Zhang et al., 2014).

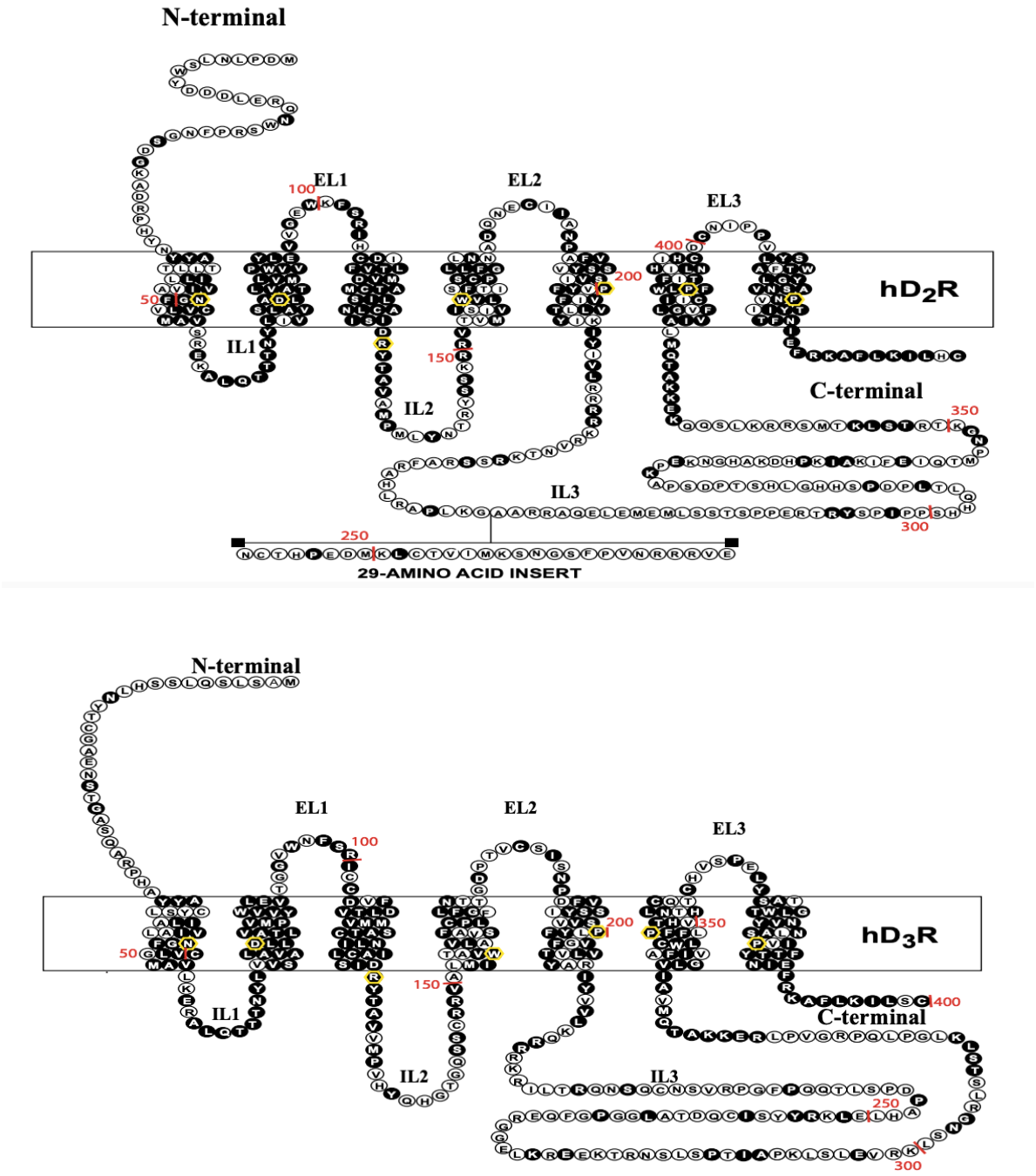


Figure 7. Secondary Structures and Amino Acid Sequences of the Human D_2R and D_3R
Black circles represent identical amino acid residues conserved between D_2R and D_3R and open circles represent the divergent residues between the two subtypes. The D_2RL and D_2RS differ by a 29 amino acid insertion in IL3. Taken from (Zhang et al., 2014).

3.1.1. Expression of DARs in the Central Nervous System

D_1R-class are mostly expressed postsynaptically on DA-receptive cells, such as GABAergic medium spiny neurons (MSNs) in the striatum. In contrast, D_2R-class are expressed both presynaptically and postsynaptically (Baik, 2013; Beaulieu & Gainetdinov, 2011). The D_1R is the most highly expressed subtype in the brain. In the CNS, D_1R-class are mainly expressed in the striatum (caudate putamen), nucleus accumbens, amygdala, substantia nigra pars reticulata, olfactory bulb, amygdala, and frontal cortex. In addition, the D_1R-class are expressed at lower levels in the thalamus, hypothalamus, hippocampus and cerebellum (Klein et al., 2019). The D_2R-class are mostly expressed in the striatum, the lateral part of the globus pallidus, core of the nucleus accumbens, ventral tegmental area, amygdala, hypothalamus, cortical areas, hippocampus and pituitary gland. In the periphery, DARs are expressed in blood vessels, kidneys, heart, retina and adrenals controlling catecholamine release and the renin-angiotensin system (Klein et al., 2019).

3.1.2. Function of DARs

The effects of DA on locomotor activity has been substantially researched. Primarily, forward locomotion is controlled by the ventral striatum through D_1R, D_2R, and D_3R activity. It has also been shown that decreased locomotor activity results from the activation of D_2R autoreceptors, while activation of postsynaptic D_2R moderately increases locomotion. Some studies have demonstrated that the synergistic interaction between D_1R and D_2R, which are segregated into the direct and indirect pathways of the striatum, as well as the activation of D_1R is required for D_2R agonists to generate maximal locomotor activity. In addition, agonist activation of the D_3R, which is expressed postsynaptically in the nucleus accumbens, results in

an inhibitory effect on locomotor activity, whereas the antagonistic effect on D_3R results in stimulation of locomotion (Jackson & Westlind-Danielsson, 1994; Missale et al., 1998).

The effect of D_1R- and D_2R-classes on drug addiction has also been extensively reviewed. Specifically, both D_1R and D_2R are shown to participate in the reinforcing properties of various drugs of abuse. For instance, drugs of abuse exert their reinforcing effects by prompting large surges of DA in the NAc that activate the direct striatal pathway via D_1R and inhibit the indirect striato-cortical pathway via D_2R (Volkow & Morales, 2015). DAR stimulation of the direct pathway directly mediates reward, while the DAR-mediated inhibition of the indirect pathway opposes aversive responses. This mechanism explains why maximal drug reward is obtained when DA binds to both the D_1R and D_2R. Specifically, in order for reinforcement to occur, the drug-induced DA increases need to be fast and adequately large to stimulate low affinity D_1R as well as the D_2R, leading to activation of the direct pathway and inhibition of the indirect pathway (Volkow & Morales, 2015). Stimulation of D_1R by endogenous DA is essential for the expression of D_2R-mediated effects on gene regulation and behaviours. A study has shown that the D_1R- and D_2R-classes mediate different aspects of reinforcing stimulus produced by cocaine (Missale et al., 1998). Particularly, D_2R-class activation appears to control the motivation to seek further cocaine reinforcement in the animal model of cocaine-seeking behaviour (Missale et al., 1998). Contrastingly, the agonistic effect on D_1R-class has been shown to mediate a reduction in the drive to seek further cocaine reinforcement, making it an appropriate target for potential therapy for cocaine addiction (Self et al., 1996).

Finally, mesolimbic and mesocortical DA has been shown to mediate learning and memory. Pharmacological studies have shown that the activation of both D_1R and D_2R in the

hippocampus has a positive effect on the retention of working memory tasks in rats, while activation of D_1R and D_2R in the prefrontal cortex improves working memory in monkeys (Arnsten et al., 2015; Missale et al., 1998). Considering that there is a lack of true agonists and antagonists that discriminate between the subtypes of D_1R- and D_2R-classes, it is difficult to investigate the role of DAR subtypes on learning and memory. However, knockout mice have helped in the clarification of the role of DARs in learning and memory. Although it is important to note that the D_1R-class effect on retention of memory is likely due to the D_5R which is highly expressed in the hippocampus compared to the D_1R (Missale et al., 1998; Tiberi et al., 1991).

3.2. Properties of D_1R-class

3.2.1. D_1R-class Expression

The D_1R is the most widespread and predominantly expressed DAR in the CNS (Dearry et al., 1990; Fremeau et al., 1991; Missale et al., 1998). Specifically, the D_1R is highly expressed in several brain regions including the frontal cortex, nucleus accumbens, dorsal striatum, substantia nigra, amygdala and olfactory tubercle (Gerfen et al., 1990). Additionally, the D_1R is expressed at lower levels in the cerebellum, limbic system, hypothalamus and thalamus (Beaulieu & Gainetdinov, 2011; Missale et al., 1998). In contrast, D_5R expression is also reported in most of these regions, but in less quantity than D_1R overall (Meador-Woodruff et al., 1992; Missale et al., 1998; Tiberi et al., 1991). Ultrastructural analysis has revealed that both the D_1R and D_5R exhibit much greater expression postsynaptically. However, there are differences in the subcellular expression, as the D_1R is mainly expressed in the dendritic spines and shafts, while the D_5R is more abundant in the proximal dendritic shafts (Bergson et al., 1995; Missale et al., 1998; Smiley et al., 1994). In particular, it has been shown that in comparison to the D_5R,

which is expressed in the dendritic shafts, the D_1R is more prominently expressed within dendritic spines of the individual pyramidal neurons (Bergson et al., 1995; Missale et al., 1998; Smiley et al., 1994). Therefore, the differences observed in the cellular and subcellular localization of the D_1R and D_5R suggests that although these receptors exhibit similar pharmacology, they are not functionally redundant (Missale et al., 1998).

3.2.2. D_1R-class Pharmacological Properties

It has not been possible to pharmacologically differentiate between D_1R and D_5R (Missale et al., 1998). However, studies reveal that the D_5R generally display higher affinity for agonists and lower affinity for antagonists in comparison to the D_1R (Tiberi & Caron, 1994). DA appears to have approximately 10-fold higher affinity for the D_5R than the D_1R (Seeman & Van Tol, 1993; Tiberi et al., 1991). Furthermore, studies have shown that the non-benzazepine antagonist compounds usually show a slightly higher affinity for the D_1R than for the D_5R, such as (+)-butaclamol and flupentixol are the most selective for the D_1R (Sunahara et al., 1991; Tiberi et al., 1991). In addition, cell lines expressing the D_5R or its rat counterpart show a higher basal AC activity than those expressing the D_1R, but agonist induced maximal activation was higher for D_1R than D_5R (Tiberi & Caron, 1994). Therefore, the pharmacological properties of D_5R which include the higher basal AC level, the higher DA affinity, and the fact that some antagonists display negative efficacy at D_5R, but not at D_1R, demonstrates that the D_5R exhibits constitutive activity (Demchyshyn et al., 2000; Tiberi & Caron, 1994).

3.2.3. D_1R-class Structural Characteristics

Although the D_1R and D_5R exhibit similar function and distribution, there are a few characteristic differences that have been reported in the genetic structure of each receptor. For instance, the D_1R-class are highly homologous within the TM domains, however the structural analysis of the D_1R and D_5R show that the IL3 and the CT are similar in size yet divergent in their amino acid sequence. In contrast, the IL1 and IL2 are highly conserved among both of these receptors (Missale et al., 1998). Therefore, any differences observed in the biological mechanisms of these receptors may be related to the IL3 and the CT regions. Additionally, various mutagenesis studies of the IL3 and CT have demonstrated the importance of these domains for G-protein coupling and binding properties of GPCRs. It has been shown that the differences in IL3 and CT sequence of the D_1R and D_5R underlie the observed distinct properties in DA potency for stimulating AC, DA binding affinity, maximal binding capacity (B_{max}), and constitutive activation (Charpentier et al., 1996; Iwasiow et al., 1999; Jackson et al., 2000; Tumova et al., 2003).

Since it is known that the IL3 regulates G protein coupling, it is possible that given the divergence in the IL3 structure of the D_1R and D_5R, each receptor may interact with different $G\alpha_{s/olf}$ isoforms under various conditions (König & Grätzel, 1994; Schulz et al., 1999; Wang et al., 2001). In addition, the IL3 and CT are characteristically rich in serine and threonine residues which can become phosphorylated, contributing to the variability in D_1R-class function (O'Dowd, 1993). Research has shown that the IL3 and CT of the D_1R-class contain protein kinase C (PKC) sites which may become phosphorylated and alter receptor responsiveness to AC activation causing facilitation of D_1R sensitization but D_5R desensitization (Plouffe et al., 2012; Rankin & Sibley, 2010). Finally, the IL3 and CT differences of the D_1R-class may determine the

distinctive protein-protein interactions with each receptor (Bermak et al., 2002; Kim et al., 2002; Zhang et al., 2007).

3.2.4. D_1R-class Canonical Signaling Pathway

GPCR signaling is mainly mediated by coupling to different G proteins and upon agonist binding to the receptor, intracellular responses are elicited depending on the type of receptor that is activated. DA downstream signaling predominantly involves G proteins, however, DAR signaling can also engage G protein-independent signaling pathways (Klein et al., 2018). The classical signaling trajectory for D_1R-class activation involves coupling to the $G\alpha_{s/olf}$ which leads to the stimulation of AC, production of the second messenger cAMP and activation of PKA (Kebabian & Calne, 1979; Kebabian & Greengard, 1971). PKA is involved in the regulation of several downstream signaling pathways and gene regulation, including DARPP-32, cAMP response element-binding protein (CREB) and ion channels (Greengard, 2001) **(Fig. 8)**.

DARPP-32 is highly expressed in MSNs and amplifies PKA signaling in the striatum to impact DA-mediated behaviours (Svenningsson et al., 2004). PKA phosphorylates DARPP-32 at threonine 34 (Thr34) and promotes the inhibitory activity of DARPP-32 towards protein phosphatase 1 (PP1). PP1 controls histone dephosphorylation, therefore inhibition of PP1 allows DARPP-32 to turn the pathway equilibrium towards a phosphorylated state which enhances the efficacy of PKA-mediated signaling (Greengard, 2001; Hemmings et al., 1984; Stipanovich et al., 2008). This results in the increase in phosphorylation of various ion channels and neurotransmitter receptors, facilitating synaptic function and plasticity. Moreover, once PKA is activated through DARPP-32, there is an enhancement of gene expression in response to D_1R-class stimulation (Stipanovich et al., 2008).

Furthermore, cyclin-dependent kinase 5 (CDK5) may phosphorylate DARPP-32 at Thr75 which prohibits the inhibition of PP1 and diminishes PKA activity (Bibb et al., 1999). DARPP-32 can also be phosphorylated by casein kinase 1 (CK1) at serine 137 (Ser137) which decreases Thr34 phosphorylation by PKA (Desdouits et al., 1995). In addition, the casein kinase 2 (CK2) phosphorylates DARPP-32 at Ser97/102 and enhances phosphorylation of DARPP-32 by PKA (Bibb et al., 1999). The CK2 signaling mechanism may be counteracted by activation of protein phosphatase 2A (PP2A) which dephosphorylates DARPP-32 at Ser97, decreasing PKA-dependent DARPP-32 signaling. It has also been shown that D_2R-class activation results in decreased PKA activation which elicits a reduction in the phosphorylation of DARPP-32 at Thr34 (Nishi et al., 2017). Specifically, D_2R-class activation also results in increased Ca^{2+} concentrations which activates calmodulin-dependent protein phosphatase 2B (PP2B, also known as calcineurin), causing dephosphorylation of DARPP-32 at Thr34 (Nishi et al., 2017).

3.2.4.1. Alternative D_1R-class Signaling Pathway

In addition to the regulation of AC activity through $G\alpha_{s/olf}$, studies have suggested that D_1R-class could couple to $G\alpha_q$ protein and regulate PLC (Sahu et al., 2009). One possible mechanism that allows D_1R-class to regulate PLC is through heterodimerization of the D_1R and D_2R in neurons. PLC catalyzes the production of IP3 which increases intracellular Ca^{2+} levels and leads to the activation of enzymes such as PP2B and calcium/calmodulin-dependent kinase II (CaMKII) (Berridge, 2016). PLC also catalyzes the production of DAG, which results in the activation of PKC (Berridge, 2016). Interestingly, the D_5R may also form a heterodimer with D_2R as it has been shown to couple to the $G\alpha_q$ followed by activation of the PLC signaling pathway and increasing Ca^{2+} levels (Sahu et al., 2009; So et al., 2009) **(Fig. 8)**. Therefore, it may

be the principal contributor to the increase in Ca^{2+} release seen in human embryonic kidney 293 (HEK293) cells, but to a lesser degree than the D_1R/ D_2R heterodimer (Hasbi et al., 2010).

3.2.4.2. D_1R class-Mediated ERK1/2 Activation

DA receptor stimulation may require specific states that only occur under the co-activation of other types of receptors and signaling pathways. One co-activated pathway converges on the ERKs, which have been shown to act as coincidence detectors that integrate the actions of DA with other neurotransmitter systems (Beaulieu & Gainetdinov, 2011). Studies have shown that both D_1R- and D_2R-classes can regulate the MAPKs, known as ERK1 and ERK2. ERK1/2 play a role in cell death and development, as well as in synaptic plasticity, leading to numerous physiological responses (Beom et al., 2004; Chen et al., 2004). Various *in vitro* and *in vivo* studies in striatal, hippocampal, PFC, amygdala and cortical neurons, as well as in HEK293 cells have confirmed that D_1R-class mainly acts to facilitate ERK1/2 activation. ERK1/2 activation by D_1R-class is also driven by the interaction with glutamate NMDARs **(Fig. 9)**.

Activation of NMDAR leads to the activation of the Ras-Raf-MEK-ERK signaling pathway (Valjent et al., 2000; Valjent et al., 2005). However, without D_1R-class activation, this activity is restrained by the striatal-enriched tyrosine phosphatase (STEP), which dephosphorylates ERK (Paul et al., 2003). PP1, which is inactivated by PKA-DARPP-32, is known to dephosphorylate and maintain the activity of STEP. Therefore, D_1R-class stimulation induces PKA function and results in PP1 inactivation, persistence of STEP phosphorylation, increased ERK phosphorylation and halts the dephosphorylation of upstream proteins of ERK cascades (Beaulieu & Gainetdinov, 2011; Valjent et al., 2000; Valjent et al., 2005). It has also been shown that the D_1R-class activation may phosphorylate NMDAR subunits, resulting in an

increase in intracellular Ca^{2+} influx which subsequently activates a variety of signaling cascades including CaMKII and the Ras-Raf-MEK-ERK pathway (Colbran & Brown, 2004; Dolmetsch et al., 2001; Pascoli et al., 2011) **(Fig. 9)**.

In addition to the effects on PKA, the D_1R-class may also act on other cAMP-regulated molecules to exert physiological actions. The guanine-nucleotide-exchange factors (GEFs), known as Epac1 and Epac2, are exchange proteins that are highly enriched in the striatum and are directly activated by cAMP (Gloerich & Bos, 2010; Missale et al., 1998b). Epac2 is involved in D_1R-class-mediated synapse remodeling and depression in rat cortical neurons (Woolfrey et al., 2009). Finally, D_1R-class also exerts cAMP-dependent effects, independent of PKA and Epac activity, which suggests that there are other cAMP-responsive molecules implicated in DAR signaling (Podda et al., 2010).

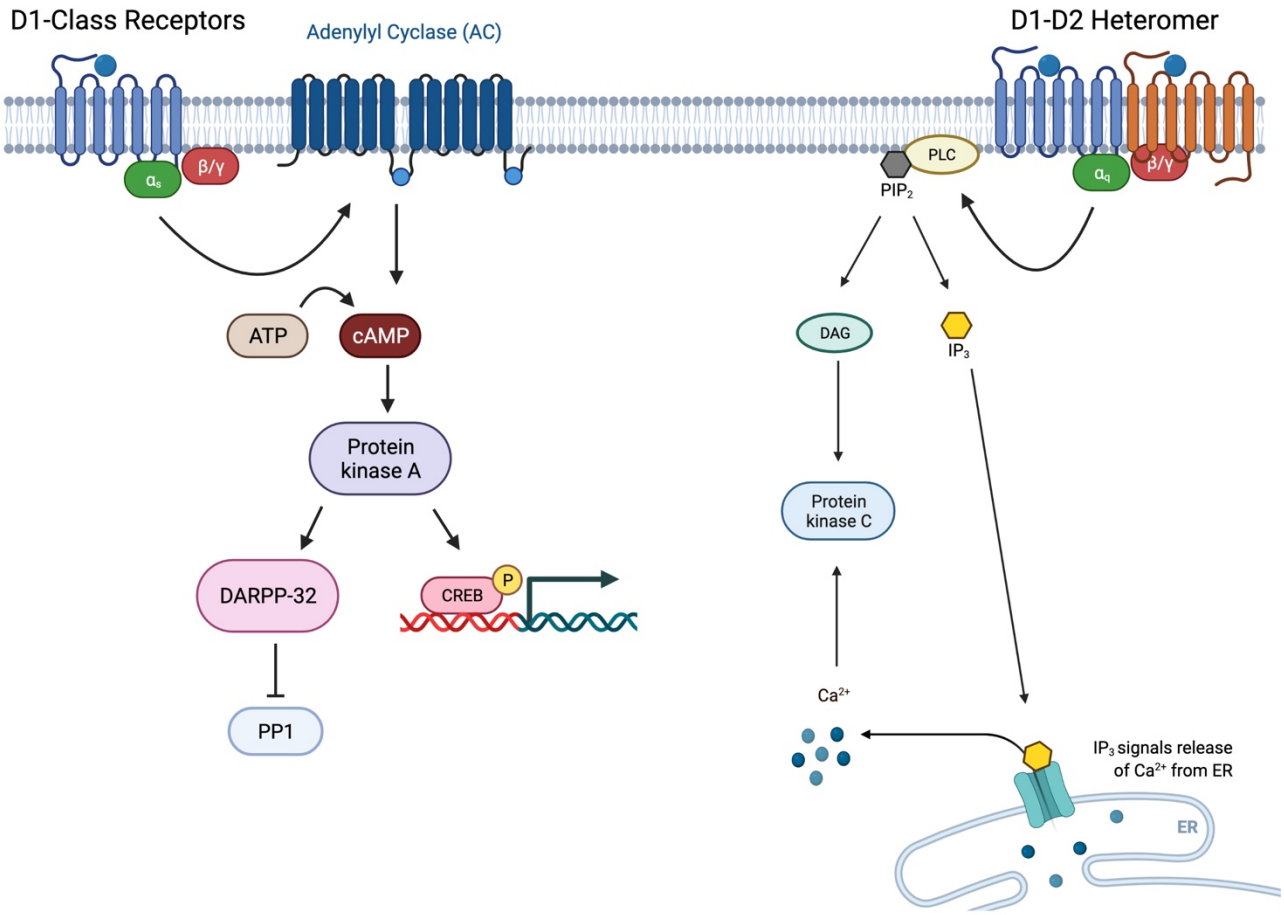


Figure 8. Schematic Representation of D_1R-class Canonical Signaling Cascade
D_1R-class primarily signal through the $G\alpha_{s/olf}$ coupled pathway. D_1R-class activate AC through $G\alpha_{s/olf}$, thereby increasing intracellular cAMP which subsequently stimulates PKA to activate the inhibitory function of DARPP-32 on PP1. PKA also phosphorylates CREB. The D_1R-D_2R heterodimer signals through $G\alpha_q$ to stimulate PLC. PLC catalyzes the hydrolysis of PIP_2 which activates DAG and leads to an increase in production of IP_3 and release of intracellular calcium from the ER. Calcium and DAG then activate PKC. Created with BioRender.com

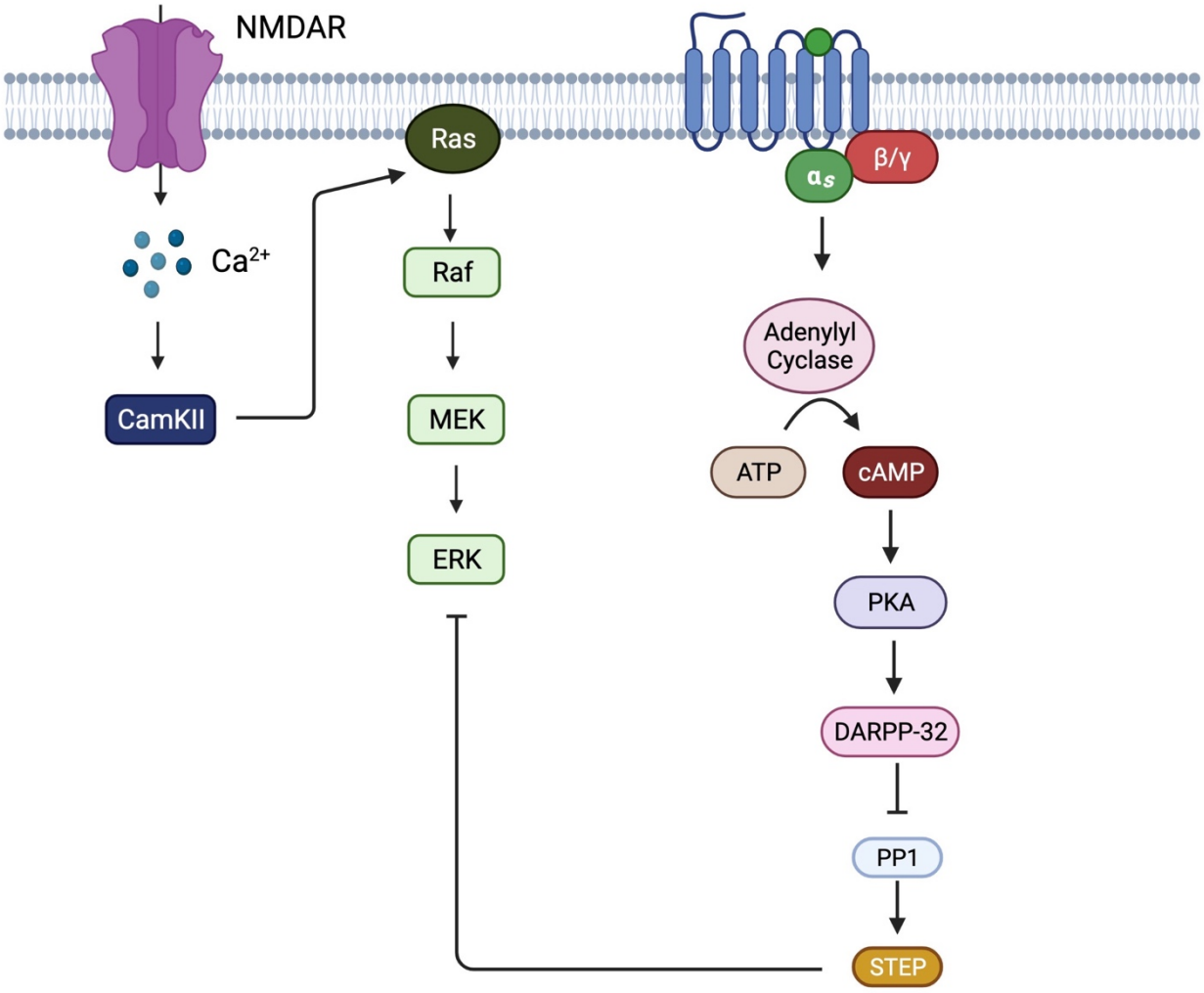


Figure 9. Schematic Representation of D_1R-class-Mediated ERK1/2 Signaling Pathway
Activation of NMDAR leads to the activation of the Ras-Raf-MEK-ERK signaling pathway, and in the D_1R-class signaling cascade, STEP is activated by PP1 to inhibit ERK activity. D_1R-class stimulation induces PKA function, PP1 inactivation and STEP phosphorylation which results in increased ERK phosphorylation and halts the dephosphorylation of upstream proteins of ERK. Created with BioRender.com

3.2.5. D_1R-class Trafficking and Interacting Proteins (DRiPs)

DA neurotransmission is regulated by a cohort of cytoskeletal, adaptor and signaling proteins called dopamine receptor interacting proteins (DRIPs) (Bergson et al., 2003). Studies have shown that D_1R-class signaling and trafficking are modulated by cytoskeleton proteins; neurofilament-M (NF-M), gamma subunit of the coatomer protein complex (gamma COP), PSD-95, as well as various adapter/chaperone proteins including the dopamine receptor interacting protein-78 (DRiP-78), calnexin, calveolin-1, N-ethylmaleimide-sensitive factor (NSF) and sorting nexin-1 (SNX-1) (Wang et al., 2008). For instance, the co-expression of the full-length NF-M with the D_1R in HEK293 cells results in greater than 50% reduction of receptor binding and cAMP accumulation (Kim et al., 2002). Also, NF-M reduces D_1R cell surface expression and promotes accumulation of the D_1R in the cytosol, but the D_1R that are co-expressed with NF-M at the cell surface are resistant to agonist-induced desensitization. The NF-M and D_1R are known to colocalize in vivo, striatum, pyramidal cells and interneurons within the frontal cortex (Kim et al., 2002). Moreover, the CT of D_1R was shown to directly bind to the γ subunit of the gamma COP coatomer complex *in vitro*, which leads to D_1R transport to the cell surface (Bermak et al., 2002). The alanine substitutions of some hydrophobic residues in the CT of D_1R was shown to result in receptor arrest in the ER (Bermak et al., 2002). Similarly, PSD-95 is a prototypical scaffolding protein which was found to interact with the CT of D_1R, *in vitro* and *in vivo*, specifically in the striatum and forebrain regions (Wang et al., 2008). The D_1R-PSD-95 interaction causes an inhibition of D_1R-mediated cAMP accumulation without changing total D_1R expression or agonist binding properties. This could be mediated by a reduction in the cell surface expression of D_1R or an enhancement in constitutive, dynamin dependent endocytosis (Zhang et al., 2007). Alternatively, PSD-95 could either modulate G-protein coupling to D_1R to

alter cAMP production and uncoupling to D_1R to activate D_1R resulting in desensitization (Wang et al., 2008; Zhang et al., 2007).

Furthermore, studies have suggested that the DRiP-78, an ER-membrane bound protein, interacts with the $F(X)^3F(X)^3F$ motif of the proximal CT of D_1R. This interaction effects D_1R transport by blocking the cargo-selection process through binding and masking an ER-export signal (Bermak et al., 2001). Another chaperone protein that resides in the ER is calnexin and it interacts with the CT of D_1R to modulate the trafficking and delivery of the newly synthesized D_1R to the cell surface (Wang et al., 2008). In contrast, calveolin-1 was shown to interact with D_1R to mediate internalization through a PKA-independent pathway and a slower process than clathrin-mediated endocytosis (Wang et al., 2008). Finally, NSF also interacts with the CT of D_1R-class to allow post-endocytic recycling of the receptor to the plasma membrane, while the SNX-1 only interacts and targets the D_5R to lysosomal degradation (Heydorn et al., 2004).

4. Overview of the Promyelocytic Leukemia Zinc Finger (PLZF) Protein

As my project focuses on the interplay between PLZF and the D_1R-class, the next section of this thesis reviews the current knowledge and research on the PLZF protein.

4.1. Discovery of PLZF

The promyelocytic leukemia zinc finger (PLZF) protein was first identified in a patient diagnosed with acute promyelocytic leukemia (APL). APL is an aggressive type of acute myeloid leukemia in which hematopoietic differentiation is hindered and promyelocytes are accumulated in the blood and bone marrow. Most cases of APL arise due to a chromosomal translocation that fuses the retinoic acid receptor alpha (RARα) gene located on chromosome 11 with the PML gene located on chromosome 15. However, abnormal cases were identified with a t(11;17) (q23;q21) translocation, which allowed for the RARα gene to be fused with a novel gene that was later cloned and identified as PLZF (Chen et al., 1993; Liu et al., 2016; Suliman et al., 2012).

4.2. Structure of PLZF

The PLZF protein, also known as ZNF145 (zinc finger protein 145) or *zbtb16* (zinc finger and bric à brac, tramtrack, and broad BTB domain containing 16), belongs to the POK (POZ and Krüppel) family of transcriptional repressors (Zollman et al., 1994) **(Fig. 10)**. In humans, the *zbtb16* gene is localized to chromosome 11q23 among a group of genes all related to the zinc finger family. It contains six exons distributed over 201 kb of DNA and holds three splice variants, all encoding functional proteins. The splice variants are composed of seven exons with shared homology between exons 3 and 6. The first and second transcripts both encode a 673 amino acid protein and are 2477 bp and 2249 bp in length. The third transcript encodes a 550

amino acid protein and is 1728 bp in length. The PLZF protein contains 673 amino acids and the C-terminus is composed of nine Krüppel-like C_2H_2 zinc fingers, which makes it a member of the Krüppel-like zinc finger protein family **(Fig. 10)**. The zinc finger protein family contains multiple cysteine (Cys) and/or histidine (His) residues that require zinc ions to stabilize their structures (Suliman et al., 2012). Zinc finger proteins are known to play crucial roles in DNA and RNA binding, RNA packaging and protein-protein interactions (Laity et al., 2001). Therefore, the zinc finger domain enables sequence-specific DNA binding to its target genes allowing PLZF to behave as a transcriptional activator or repressor (Li et al., 1997). In addition, the N-terminal 118 amino acids contains a drosophila-like bric à brac, tramtrack, and broad (BTB)-complex domain. This N-terminus segment of the protein is also known as the poxvirus and zinc finger (POZ) domain and allows for protein dimerization as well as interactions with other proteins to form multi-protein complexes (Bardwell & Treisman, 1994; Yoshida et al., 1999). The middle region of PLZF has a middle second repression domain (RD2) which allows for regulation of the transcriptional activity of the protein (Kang et al., 2003) **(Fig. 10)**.

Evidently, the PLZF protein structure reveals that it is a DNA-binding zinc finger transcription factor and it has been shown to impede the differentiation of promyelocytes resulting in leukemia. Therefore its role in various biological activities maintaining cellular proliferation and differentiation has been extensively investigated by researchers (Shaknovich et al., 1998). However, PLZF expression is not limited to stem cells and early progenitor cells as recent studies have shown that it is also expressed in several other cell types such as CNS cells, hematopoietic cells in the bone marrow, myocytes in the heart and skeletal muscles, glandular cells in the gallbladder, respiratory epithelial cells, endometrial stroma in the uterus, islets of

Langerhans in the pancreas, glomeruli and renal tubules, glandular cells of the gastrointestinal tract, prostate and endocrine glands (Uhlén et al., 2005).

4.3. PLZF Animal Models

To better analyze the role of PLZF in distinct biological systems and cellular functions, an animal model of PLZF (*zbtb16*) gene knockout was developed by Barna and colleagues at Cornell University. PLZF$^{-/-}$ mice were generated by replacing the second exon of full-length PLZF with the neomycin resistance gene. These mice presented an array of deformities including musculoskeletal-limb defects with changes in the developmental fate of vertebral segments, deformed cartilage and skeletal patterning and changes in formation of digits. These defects are a result of inhibition of apoptosis in early progenitor cells of the hindlimb, perhaps due to *Hox* gene regulation. *Hox* genes are major regulators of animal development and are known to specify segment identity in an embryo. Moreover, PLZF was found to be crucial for spatial colinear expression of *HoxD* genes in the hindbrain. The depletion of PLZF levels during pluripotent osteoblastic differentiation of the human mesenchymal stem cells was also found to result in spinal bone formation (Barna et al., 2000; Barna et al., 2002; Barna et al., 2005). In addition, the PLZF$^{-/-}$ mice show partial sterility as a direct result of significantly impaired spermatogenesis. The characteristic decrease of spermatozoal maturation in the seminiferous tubules is a result of the increase in apoptosis of spermatogonial cells (Kelly & Daniel, 2006). This phenomenon suggests a central role for PLZF in governing the undifferentiated state of spermatogonial cells (Buaas et al., 2004).

Lastly, researchers observed that the PLZF$^{-/-}$ mice were more susceptible to viral infections. In a study by Xu and colleagues, neonatal wild-type and PLZF$^{-/-}$ mice were exposed

to the fatal Semliki Forest virus or encephalomyocarditis virus (Xu et al., 2009). Both genotypes of mice were pretreated with interferon (IFN) prior to infection and the mice lacking PLZF expression died after six days of infection, whereas the wild-type neonatal mice had a survival rate exceeding three weeks post-infection. This study demonstrates the importance of PLZF in regulation of the IFN-mediated anti-viral innate immune response in vivo (Xu et al., 2009). Hence, the animal model of PLZF gene knockout reveals the dynamic role of PLZF in regulation of various cellular functions such as the maintenance and proliferation of specific stem cells.

4.4. Cellular Localization of PLZF

To better understand the dynamic role of PLZF, research has extensively focused on the localization of PLZF in the cell. Primarily, the structure of PLZF contains an N-terminal BTB/POZ domain, which has been shown to mediate homo- and hetero-dimerization, nuclear localization as well as direct binding of corepressors (Lin et al., 2013). Therefore through analysis of the structure of PLZF, it has been found that PLZF achieves transcriptional repression through recruitment of nuclear receptor corepressors such as N-CoR or SMRT as well as histone deacetylase (HDAC) complexes via its POZ domain (Lin et al., 2013).

PLZF is able to activate gene expression and is localized inside the nucleus within nuclear speckles. Nuclear speckles or interchromatin granule clusters are involved in pre-mRNA processing and contain numerous transcription factors (Galganski et al., 2017). Various studies have shown that PLZF may function as a transcriptional repressor or activator depending on the molecular context and genomic framework of the target loci (Liu et al., 2016). Thus, there is evidence showing that PLZF loses its transcriptional repression as it is being shuttled from the inside of the nuclear speckles to the outside (Koken et al., 1997; Nanba et al., 2003).

For instance, a study investigated the proteolytic cleavage of the heparin-binding EGF-like growth factor (HB-EGF) by treating human fibrosarcoma HT1080 cells with phorbol-ester TPA, an activator of PKC (Nanba et al., 2003). Research has shown that the cleavage of proHB-EGF is required for the epidermal growth factor receptor transactivation by GPCR signaling which is implicated in various pathologies such as cardiac hypertrophy and cystic fibrosis (Prenzel et al., 1999). This metalloprotease cleavage of the membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF) releases its soluble form, known as HB-EGF-C (Nanba et al., 2003). The soluble form, HB-EGF-C then binds the zinc finger motif of PLZF and allows for its nuclear export to the cytoplasm. Therefore, the nuclear PLZF levels decreased in the TPA-treated HT1080 cells, which subsequently resulted in increased cyclin A2 levels and impeded entry of S-phase in the cell cycle, thereby suppressing cell growth (Nanba et al., 2003). In contrast with these findings, various studies have shown that PLZF expression inside nuclear speckles and its transcriptional activity were increased in response to far-infrared therapy, retinoic acid (RA) and interferon IFN treatment (Hsu et al., 2012; Koken et al., 1997).

4.5. The Role of PLZF in Regulation of Cell Cycle

The cell cycle is an extensive and intricate process involved in the growth and proliferation of cells, regulation of DNA damage repair, and diseases such as cancer (Schafer, 1998). It involves various regulatory proteins that guide the cell through stages referred to as G1, S, G2, and M phases (Schafer, 1998). PLZF is one of these regulatory proteins as it has been shown to negatively regulate the cell cycle and apoptosis of numerous cell types (Melnick et al., 2000). Expression of PLZF results in the aggregation of cells in the G0/G1 phase and augments the prevalence of apoptosis (McConnell et al., 2003). Specifically, PLZF binds to the promoters

of key cell cycle and proproliferative genes, such as c-Myc and cyclin A2 to repress their expression and result in cell cycle arrest as well as growth suppression (Costoya et al., 2008; McConnell et al., 2003). Furthermore, PLZF also directly activates the regulated in development and DNA damage responses 1 (Redd1), which inhibits mammalian TOR complex 1 (mTORC1) and results in the arrest of late G1 phase (Kolesnichenko & Vogt, 2011; Nowosad et al., 2020) **(Fig. 11)**.

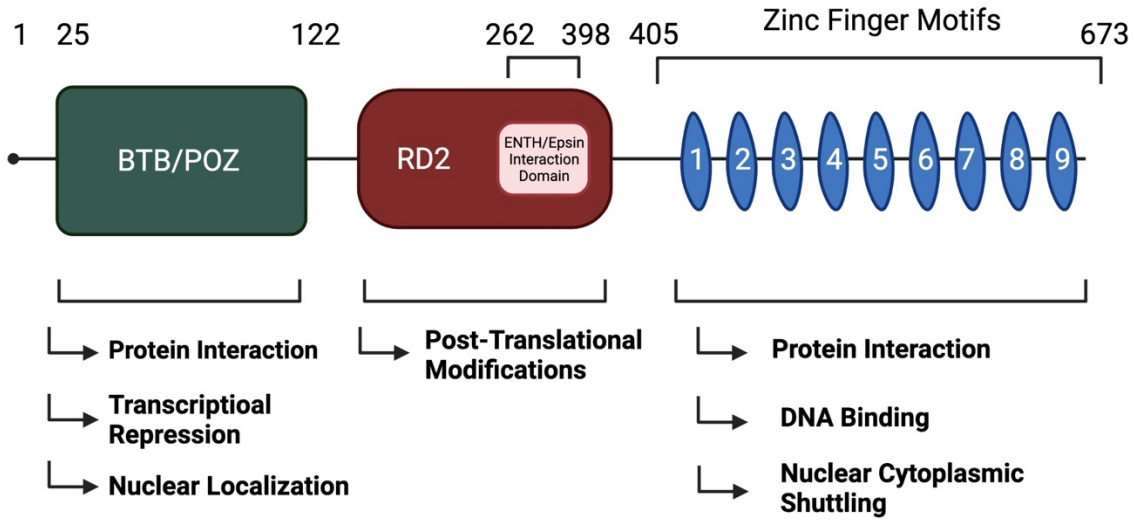


Figure 10. Schematic Diagram of PLZF Protein Structure
The three functional domains of PLZF include: The BTB/POZ domain, the RD2 domain, The ENTH domain and the zinc finger domain. The diagram shows the full length primary transcript of PLZF and the important functions of each domain are listed. The NT contains the BTB/POZ domain and the zinc fingers are located within the CT. Created with BioRender.com

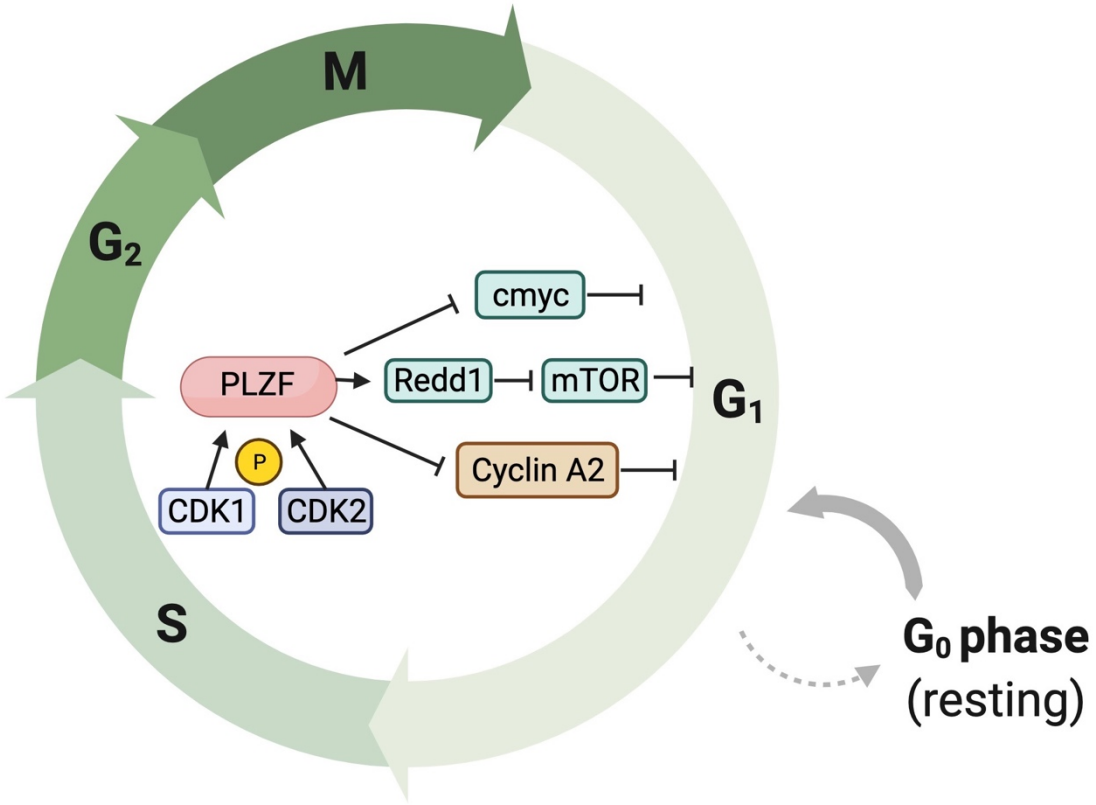


Figure 11. The Role of PLZF in Regulation of the Cell Cycle
PLZF regulates the cell cycle by inhibiting cellular proliferation in G_0/G_1 phase. CDK1 and CDK2 phosphorylate PLZF to regulate its stability. PLZF inhibits the activity of cyclin A2 and cmyc through binding to the promoters of these proproliferative genes, or activates Redd2 to antagonize mTOR1. This results in cell cycle arrest or apoptosis. Created with BioRender.com

4.6. The Role of PLZF in Stem Cell Self-Renewal

Transcription factors have been presented as major regulators of stem cell homeostasis as they integrate intrinsic and extrinsic cues. Evidently, PLZF is a master transcriptional regulator, which has been shown to control stem cell homeostasis by sustaining the balance between progenitor pools and generation of numerous differentiated cells. Studies have revealed that PLZF maintains growth, self-renewal and differentiation of stem cells, and specifically plays a well-recognized role in hematopoietic, spermatogonial, mesenchymal and neural progenitor cells (Reid et al., 1995; Dai et al., 2002; Liu et al., 2016).

4.6.1. PLZF and Hematopoietic Stem Cells

Primarily, within the hematopoietic tissue, PLZF is highly expressed in undifferentiated hematopoietic stem cells (HSCs) and progenitors, but its expression is downregulated in differentiated cells, which indicates that PLZF is involved in stem cell maintenance and self-renewal (Poplineau et al., 2019). For instance, a study revealed that forced expression of PLZF in vitro immortalizes HSCs and myeloid progenitors (Ono et al., 2013). However, the downregulation of PLZF was shown to suppress the mixed lineage leukemia (MLL)-fusion-induced leukemic transformation of HSCs in vitro and in vivo (Ono et al., 2013).

PLZF shifts the balance against differentiation by repressing the expression of myeloid transcription factors, including growth factor independence-1 (GFI-1), CCAAT/enhancer binding protein α (C/EBPα) and lymphoid enhancer-binding factor 1 (LEF1), and activate the negative regulators dual specificity phosphatase 6 (DUSP6) and inhibitor of DNA binding 2 (ID2) (Doulatov et al., 2009). Interestingly, induction of ERK1/2 by myeloid cytokines creates a stress response which leads to nuclear export and inactivation of PLZF, generating an increase in

mature cell production (Doulatov et al., 2009). These findings demonstrate the role of PLZF in self-renewal of myeloid progenitors and HSCs.

Moreover, the PLZF/RARα fusion protein interacts with the Polycomb protein Bmi-1 to recruit the polycomb-repressive complex 1 (PRC1) to RA response elements and mediate leukemic transformation (Boukarabila et al., 2009). Contrarily, the RARα/PLZF fusion protein acts as a modifier oncogene by recruiting HDAC1 and causing H3 His deacetylation at the C/EBPα locus (Girard et al., 2013; Pulikkan et al., 2017). This results in the inhibition of C/EBPα activity and subverts myeloid differentiation to activate self-renewal of myeloid progenitors (Girard et al., 2013).

4.6.2. PLZF and Spermatogonial Stem Cells

Spermatogonial stem cells (SSCs) are located in the basal membrane of seminiferous tubules, and are responsible for spermatogenesis via differentiation into fully functional sperm cells. The undifferentiated spermatogonia population are called spermatogonial progenitor cells (SPCs) and are an essential factor of male fertility. As demonstrated in the animal model, $PLZF^{-/-}$ mice are infertile because of the limited numbers of normal spermatozoa in young mice. PLZF plays a significant role in maintaining spermatogonial self-renewal by behaving as an intrinsic factor and a biomarker for SPCs. Evidently, PLZF is pivotal for the maintenance and self-renewal of SPCs yet the underlying mechanism of PLZF in regulation of SPC fates is not fully understood.

Primarily, studies have shown that SPCs lacking PLZF have enhanced mTORC1 activity (Hobbs et al., 2010). mTORC1 is a key mediator of cell growth as it is a signaling complex that promotes protein translation by phosphorylating components of the translation machinery (Ma &

Blenis, 2009). Irregular mTORC1 activation in the SPCs of PLZF$^{-/-}$ mice results in negative feedback that inhibits their response to glial cell-derived neurotrophic factor (GDNF), a growth factor that is crucial for SPC self-renewal (Hobbs et al., 2010). When PLZF is present, it has been shown to activate Redd1 which downregulates the aberrant mTORC1 and membrane-associated non-receptor tyrosine kinases (Src) family kinases, as well as activates the PI3K/protein kinase B (AKT) signal pathways (Hobbs et al., 2010). Therefore, the mTORC1-PLZF functional interaction is critical in maintaining the spermatogonial pool by balancing SPC growth with self-renewal.

Another proposed mechanism is that PLZF directly inhibits the transcription of the receptor tyrosine kinase (c-kit), a feature of spermatogonial differentiation (Filipponi et al., 2007). Specifically, the c-kit receptor tyrosine kinase plays a critical role in the postnatal stages of spermatogenesis (Filipponi et al., 2007; Song et al., 2020). A point mutation responsible for phosphatidylinositol 3-kinase docking in the c-kit gene impedes the early stages of spermatogenesis and eliminates DNA synthesis in differentiating spermatogonia, resulting in sterility (Filipponi et al., 2007; Tu et al., 2018). Research has essentially shown that a 3-bp mutation in the PLZF binding site terminates the c-kit promoter responsiveness to PLZF repression (Filipponi et al., 2007). Thus, PLZF knockout mice exhibit a notable increase of c-kit expression in their undifferentiated spermatogonia, meaning that PLZF sustains the pool of SSCs through direct transcriptional repression of c-kit (Azizi et al., 2020; Filipponi et al., 2007; Rossi, 2013). Therefore, this data reveals that PLZF plays a critical role in the self-renewal of the spermatogonial pool.

4.6.3. PLZF and Neural Stem Cells

The formation of neural circuits within the developing central nervous system (CNS) requires meticulous spatial and temporal generation of distinct classes of neurons and glia from the multipotent neural stem cells (NSCs) and neural progenitor cells (NPCs) (Gaber et al., 2013; Liu et al., 2016; Sobieszczuk et al., 2010). The key aspect of this process is the propensity of NPCs to self-renew in a manner that enables early-born cells such as neurons to form while sustaining an adequate progenitor pool to generate later-born cell types such as glia (Gaber et al., 2013). Studies have revealed that PLZF is expressed in dividing progenitors yet downregulated during neural differentiation.

For instance, PLZF maintains neural progenitors by upregulating fibroblast growth factor receptor 3 expression and the downstream STAT3 pathway activity. In the developing spinal cord, specifically the early neural plate and tube, NPCs are organized in a proliferative neuroepithelial sheet and sustained by the mitogenic actions of growth factors like fibroblast growth factors FGFs (Gaber et al., 2013). Throughout neural development, FGFs are present in neural tissues where they act through the FGFRs expressed by NPCs. Subsequently, ligand binding to FGFRs activates downstream signaling cascades such as the STAT3 pathway to promote cell division and inhibit neuronal differentiation (Gaber et al., 2013). In contrast, it is known that reduced PLZF activity undermines neural progenitor maintenance, leading to neuronal differentiation. For instance, a study has shown that the BTB Domain Containing 6 (BTBD6), an adaptor protein for the cullin-3 (Cul-3) ubiquitin ligase complex, binds to PLZF to promote the translocation of PLZF from the nucleus to the cytoplasm for ubiquitination and degradation, which ultimately leads to neuronal differentiation (Sobieszczuk et al., 2010).

4.7. Regulation of PLZF

The stability or transcriptional activity of PLZF is precisely regulated by post-translational modifications, upstream factors, and multiprotein complexes. Moreover, PLZF can regulate target genes through homodimerization **(Fig. 12)**.

4.7.1. Post-translational Modifications of PLZF

4.7.1.1. Acetylation of PLZF

The PLZF protein has been shown to undergo various posttranslational modifications after its biosynthesis in different regions. Evidently, PLZF acts as a transcriptional repressor by binding to the specific DNA sequence 5'-TACTGTAC-3' found in the promoter region of its target genes. PLZF has also been shown to undergo acetylation, which involves the reversible addition of an acetyl group to lysine residues in the protein. For both in vivo and in vitro conditions, the lysine residues in zinc finger 9 of PLZF can be acetylated by p300 histone acetyltransferase **(Fig. 12)**. Therefore, acetylation prevents PLZF from binding to its targets genes and consequently loses its transcriptional repression. This process does not alter the stability of PLZF and its binding capacity to other proteins as it specifically targets the zinc fingers of PLZF which are essential in DNA binding (Guidez et al., 2005).

4.7.1.2. Ubiquitination of PLZF

Moreover, PLZF is subject to the posttranslational modification known as ubiquitination, which is mediated by the highly conserved ubiquitin protein. Ubiquitin targets various proteins to direct them to proteasomal degradation in the cell. Studies have shown that the BTB/POZ domain of PLZF binds BTBD6, which is a ubiquitin ligase adaptor protein for the Cul-3

ubiquitin E3 ligase complex **(Fig. 12)**. Upon binding of the ubiquitin to PLZF, the protein is exported from the nucleus to degradation. This mechanism is crucial in differentiation of NPCs as the removal of PLZF allows for recovery of the pro-neural gene neurogenin1 expression during the early stages of neurogenesis (Sobieszczuk et al., 2010).

4.7.1.3. Phosphorylation of PLZF

In addition, studies have shown that PLZF is phosphorylated in vitro by cyclin-dependent kinase 1 (CDK1), also known as cdc2 or Cyclin-B **(Fig. 12)**. The phosphorylation of PLZF by CDK1 directly regulates its function by altering its DNA-binding ability to the promoters of various transcription factors such as c-Jun and GATA1 (Ball et al.,1999). Another in vitro study revealed that PLZF is also phosphorylated by cyclin-dependent kinase 2 (CDK2) at the threonine residue 282 and serine residue 197. However, contrary to CDK1-mediated phosphorylation which modulates PLZF transcriptional regulation, CDK2 phosphorylation elicits the ubiquitination and degradation of PLZF without affecting its nuclear localization, thereby indirectly regulating its function (Costoya et al., 2008). Lastly, the mitogen-activated protein kinase 8 (JNK) which is activated by the interferon receptors also appeared to phosphorylate PLZF at serine residue 76 in vitro. This phosphorylation produced an increase in transcriptional activity and binding of PLZF to the promoters of interferon-stimulated genes (ISGs). Unlike the early studies which have appointed PLZF as a transcriptional repressor, some findings have demonstrated that PLZF can behave as a transcriptional activator through induction of ISGs and elicitation of the immune response (Xu et al., 2009).

4.7.1.4. Sumoylation of PLZF

Finally, PLZF can be sumoylated by small ubiquitin-like modifier (SUMO) proteins, which reversibly attach to target proteins to modulate their functions. In an *in vivo* study by Kang and colleagues, they observed that the endogenously expressed PLZF in human promyelocytic leukemia HL-60 cells was modified by conjugation with SUMO-1 **(Fig. 12)**. Results showed that PLZF colocalizes with SUMO-1 in the nucleus of transfected human embryonic kidney (HEK) 293T cells. SUMO-1 binds to the lysine 242 residue found in the RD2 domain of PLZF to abolish the DNA-binding activity of PLZF, which activates cyclin-A2 and consequently reduces the transcriptional repression on cell cycle progression (Kang et al., 2003). Another study has shown that metadherin, a protein involved in the metastasis of various cancer types, binds to the sumoylation sites in the RD2 terminal of PLZF while inside nuclear bodies. This association remarkably impeded the ability of PLZF to bind the *c-myc* promoter and diminished its transcriptional repression. Both proteins were also shown to colocalize to nuclear bodies containing HDACs and form complexes which results in an opposite transcriptional outcome by promoting PLZF-mediated repression (Thirkettle et al., 2009). Therefore, post-translational modifications of PLZF leads to changes in the characteristic functions of the protein such as the transcriptional fate. In addition to post-translational modifications, any interacting proteins with PLZF may result in changes in the downstream functions of the protein through signaling, growth-regulatory and differentiation pathways.

4.8. Upstream Factors Regulate PLZF

The transcriptional activity of PLZF is finely regulated by upstream factors, such as Evi-1, Cux1, and FOXO3a. A study has revealed that the Evi-1, an invasive protooncogene in human

leukemia, activates the PLZF promoter and leads to transcription (Takahashi & Licht, 2002; Yuan et al., 2015). Evi-1 binds to 140/-130 Evi-1-like site in the PLZF promoter which also leads to tissue-specific expression of PLZF in undifferentiated myeloid cells (Takahashi & Licht, 2002; Yuan et al., 2015). In addition, PLZF is a transcriptional target for the Cux-1 repressor. In colorectal cancer cells, Cux-1 interacts with multiple DNA-binding sites in the 5'-UTR and promoter of the PLZF gene (Fréchette et al., 2010) . Lastly, the phosphorylated FOXO3a transcription factor, downstream of PTEN-PI3K-AKT signaling directly binds to the promoter of the PLZF gene to inhibit tumorigenesis in both prostate and pancreatic cancer (Cao et al., 2013; Zhang et al., 2020) **(Fig. 12)**.

4.9. PLZF Multiprotein Complexes

The stability or activity of PLZF can also be regulated through the formation of specific protein-protein complexes. Therefore, there are direct targets and cofactors of PLZF, which serve to regulate its activity in diverse biological processes and developmental contexts.

4.9.1. PLZF Protein-Promoter Binding

PLZF behaves as a transcriptional activator or repressor by directly binding to the specific DNA sequence of target genes through the nine Kruppel-like zinc fingers. In gallbladder cancer (GBC), PLZF expression has been shown to significantly reduce cell proliferation, migration and invasion as it highly increases the mRNA transcription of interferon-induced protein with tetratricopeptide repeat 2 (IFIT2) (Shen et al., 2018). Therefore, PLZF acts as a tumour biomarker in GBC by increasing the STAT1 protein level, which increases IFIT2 and ultimately promotes cellular apoptosis and suppresses tumour proliferation (Shen et al., 2018).

Other target genes that PLZF activates includes redd1which leads to an attenuation of mTORC1 activity and subsequently eliminates the negative feedback to growth factor receptors like GDNF which facilitates the self-renewal of the spermatogonial pool (Kolesnichenko & Vogt, 2011). Conversely, PLZF has been shown to repress genes such as smooth muscle α-actin, an important component of the cytoskeleton. This repression results in a change of cellular shape and substantial reorganization of the cytoskeleton, which are likely aspects of tumour suppression (Kolesnichenko & Vogt, 2011). Additionally, PLZF directly binds *Hoxb2* and the highly conserved human (pro)renin receptor (RER), involved in CNS development (Ivins et al., 2003; Seidel et al., 2011). In many cases, PLZF behaves as an activator or repressor of various target genes to play a role in cell proliferation, self-renewal and differentiation **(Fig. 12)**.

4.9.2. PLZF Protein-Protein Interactions

Another level of regulation is attained through protein-protein interactions and the formation of multiprotein complexes. There are several proteins that have been reported to regulate the activity of PLZF through interacting and forming multiprotein complexes with PLZF. Primarily, PLZF is known to repress transcription by recruiting a histone deacetylase through the SMRT-mSin3-HDAC-NCoR corepressor complex (Choi et al., 2014; David et al., 1998). HDACs contribute to the transcriptional repression of PLZF by deacetylating histones to result in alterations in chromatin modeling and play a critical role in human leukemogenesis (Choi et al., 2014; David et al., 1998).

Furthermore, PLZF has been reported to interact with Epsin 1 through the epsin NH_2-terminal homology (ENTH) domain, found in the region containing amino acid 262-673 of PLZF. This interaction has been revealed to be necessary for PLZF-mediated nucleocytoplasmic

shuttling of epsin (Hyman et al., 2000). There are numerous other cofactors of PLZF, including GATA1 (Labbaye et al., 2002), GATA2 (Tsuzuki & Enver, 2002), RAR (Martin et al., 2003), and Cul-3 (Mathew et al., 2012), which are involved in APL and HSC differentiation; Bmi1 (Barna et al., 2002) and Hox5 (Xu et al., 2013), which are involved in limb morphogenesis; cdc2 (Ball et al., 1999) and $G\alpha_o$ (Won et al., 2008), which are involved in cell cycle and cell proliferation; and TIMP1 (Seung et al., 2007) and nuclear LYRIC/AEG1 (Thirkettle et al., 2009) which are involved in apoptosis during tumorigenesis (Liu et al., 2016) **(Fig. 12)**.

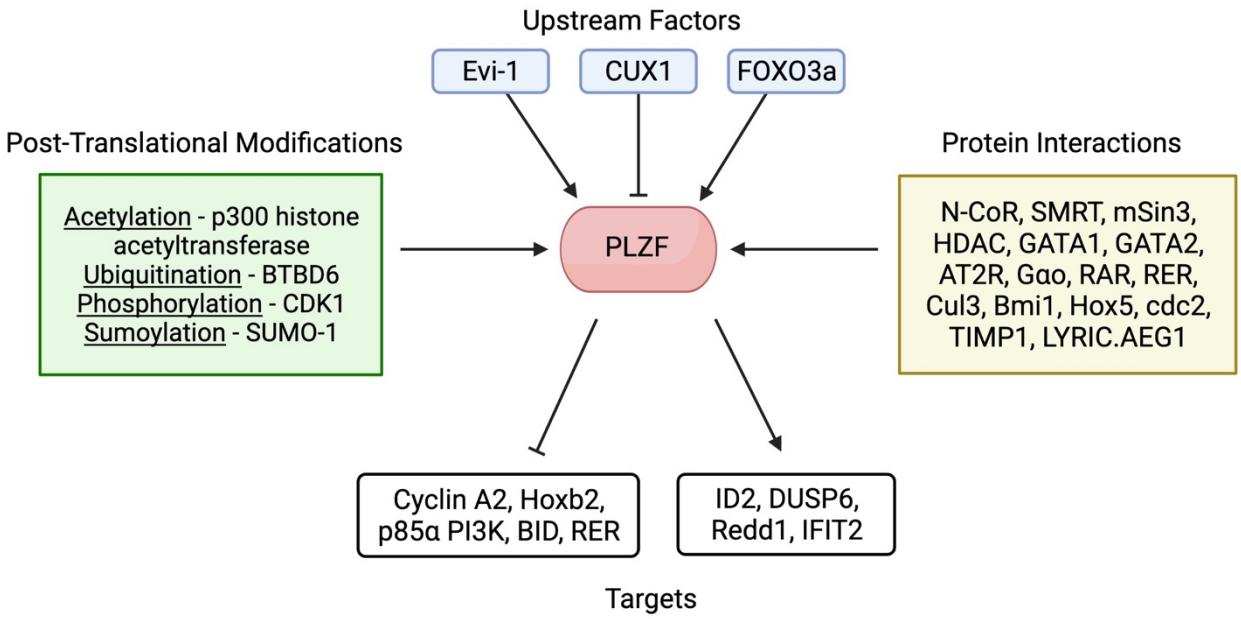


Figure 12. Schematic Representation of PLZF Regulation and Signaling Pathways
PLZF functions as a transcriptional repressor or activator by direct binding to DNA sequences of target genes through its nine zinc fingers, which affects cell proliferation, differentiation and self-renewal. The transcriptional activity or stability of PLZF is regulated by post-translational modifications, protein-protein interactions and upstream factors.

4.10. PLZF and Receptor Interactions

4.10.1. PLZF and Angiotensin II Type 2 Receptor

The GPCR angiotensin II (Ang II) type 2 receptor (AT_2R) plays important roles in the development of cardiovascular diseases including hypertension, cardiac hypertrophy and ischemic heart disease (Senbonmatsu et al., 2003). A study by Senbonmatsu et al., describes a novel signaling pathway for PLZF in regulation of AT_2R signaling in CHO cells. Results reveal that PLZF binds to the CT of AT_2R and upon Ang II stimulation, both proteins internalize causing AT_2R to be perinuclear and PLZF to be translocated to the nucleus (Funke-Kaiser et al., 2010; Seidel et al., 2011; Senbonmatsu et al., 2003).

The nuclear PLZF then activates the p85α PI3K gene leading to protein synthesis activation. This mechanism has been shown to contribute to cardiac hypertrophy elicited by chronic Ang II stimulation or pressure overload (Funke-Kaiser et al., 2010; Seidel et al., 2011; Senbonmatsu et al., 2003). The results from this study present an interesting purpose for PLZF in regulation of a $G_{i/o}$- class GPCR (Senbonmatsu et al., 2003). Primarily, it is known that the cytosolic CT of various GPCRs regulate receptor endocytosis by modulating phosphorylation and β-arrestin binding (Ferguson, 2001). However, in the absence of PLZF, other studies have shown that Ang II stimulation induced translocation of AT_1R to the perinuclear region in association with β-arrestin (Ferguson, 2001). In this study, cytosolic PLZF is bound to epsin 1 to initiate the slower internalization of AT_2R without the association of β-arrestin (Hyman et al., 2000).

The use of genistein, a tyrosine kinase inhibitor, resulted in the prevention of AT_2R-PLZF co-localization and PLZF translocation to the nucleus. Thus, it was found that Ang II stimulation induced a PLZF tyrosine phosphorylation which was responsible for the nuclear

translocation of PLZF (Senbonmatsu et al., 2003). Specifically, the phosphorylation of PLZF at Y669F was deemed responsible for initiating the signaling mechanism observed in this study, especially because the inactive PLZF mutant (Y669F) abolished the p85α PI3K gene activation as well (Senbonmatsu et al., 2003). It is known that the activation of the regulatory subunit p85α relies on growth factor receptors such as FGF or insulin-like growth factor (IGF) to facilitate the growth factor-mediated cardiac hypertrophic pathway (Seidel et al., 2011; Senbonmatsu et al., 2003).

4.10.2. PLZF and (Pro)Renin Receptor (RER)

Similar to the AT_2R, the human renin/prorenin receptor (RER) is involved in the renin-angiotensin system (RAS), specifically in cardiac and renal end-organ damage (Schefe et al., 2006; Schefe et al., 2008). However, unlike the AT_2R, RER is composed of a single transmembrane domain and specifically binds prorenin and renin (Schefe et al., 2006; Schefe et al., 2008). The RER is expressed in the brain, heart, kidney, liver and pancreas (Xu et al., 2016). In a study by Schefe et al., PLZF was found to directly interact with the C-terminal domain of the RER by yeast two-hybrid screening and co-immunoprecipitation (co-IP) (Schefe et al., 2006; Schefe et al., 2008). Similarly to the AT_2R, PLZF was translocated to the nucleus upon RER activation by renin, but PLZF repressed the transcription of the RER itself, creating a short negative feedback loop (Schefe et al., 2006; Schefe et al., 2008). The activation of RER by renin also activates the promoter activity and transcription of a second downstream target gene, the p85α subunit of the PI3K, a protein involved in stimulation of protein synthesis and cardiac hypertrophy (Schefe et al., 2006, Schefe et al. 2008; Xu et al., 2016).

4.10.3. PLZF and $G\alpha_o$ Subunit

A study by Won et al. also used the yeast two-hybrid system to screen for $G\alpha_o$-interacting proteins in the human fetal brain and PLZF was found to specifically bind to the $G\alpha_o$ subunit (Won & Ghil, 2009; Won et al., 2008). G_o is one of the most abundant G proteins expressed in the brain but the effect on AC is not clear. Initial reports indicated that G_o did not inhibit AC and more recent studies demonstrate its ability to inhibit the AC1 isoform, but not the AC5 or AC6 isoforms (De Oliveira et al., 2019). Results show that the activation of the $G_{i/o}$-coupled cannabinoid (CB) receptor significantly increased the PLZF-mediated cell growth arrest in HL60, the human APL cell line (Won et al., 2008). Evidently, PLZF induces differentiation in neurons and neuroblastoma cell lines as they express $G\alpha_o$ proteins (Jordan et al., 2005). Therefore, since studies have revealed that the activation of CB also promotes neuronal differentiation, it is possible that the CB-$G_{i/o}$ – PLZF signaling complex sustains neuronal differentiation, through inhibition of the cyclin A expression and subsequent blockade of cell proliferation (Jordan et al., 2005).

5. PLZF in the Central Nervous System

Numerous studies reveal the role of PLZF in the maintenance of neural progenitor cell proliferation and neuronal differentiation during development. However, there is limited information regarding its regulation and possible function in the brain and CNS diseases. Animal studies using the spontaneous *luxoid* (*lu*) mutant of *zbtb16* in mice demonstrate the importance of PLZF in skeletal development and germ cell self-renewal (Lin et al., 2019; Usui et al., 2021). A single nucleotide variant (c.1849A>G; p.Met617Val) in the C2H2-type zinc finger domain of *zbtb16* has been identified as a causative mutation for skeletal defects, genital hypoplasia,

microcephaly and severe mental retardation, implying the involvement of PLZF in brain development (Fischer et al., 2008; Lin et al., 2019; Usui et al., 2021).

5.1. PLZF in the Developing CNS

The development of the cerebral cortex is a complex and critical process which involves NSC proliferation, differentiation, cell fate determination and cell migration. Any defects in the proper balance between NSC self-renewal and neurogenesis results in brain malformations. Therefore, it is essential to observe the effects of PLZF on brain structures, functions and behaviour. A study by Lin et al. has revealed that PLZF is involved in the formation of deep layer cortical neurons. Researchers showed that PLZF expression was detected as early as embryonic day (E) 9.5 in $Pax6^+$ cells in the mouse brain and was completely abolished before cortical neurogenesis in the telencephalon, the most highly developed anterior region of the forebrain (Avantaggiato et al., 1995; Lin et al., 2019; Usui et al., 2021). In addition, the loss of PLZF resulted in a smaller cerebral cortex with a decrease in the number of $Tbr1^+$ marker for cortical deep layer neurons (Lin et al., 2019). Lastly, loss of PLZF resulted in the dysregulation of the *Mash1* proneural gene expression, microcephaly and impairment of recognition memory (Lin et al., 2019). Therefore, since PLZF promotes NPC proliferation and decreases neuronal differentiation, loss of PLZF in the developing CNS results in the reduction of the NSC/NPC pool and a smaller cerebral cortex with a specific decrease in the number of neurons in layer VI of the cerebral cortex (Lin et al., 2019).

Moreover, the role of PLZF in social cognitive behaviours and neocortical development was also established recently in a study that examined the mechanisms underlying the behavioural phenotypes in the young adult brain of *zbtb16* KO mice. Histological analyses

revealed impairments in thinning of the neocortical layer VI and a reduction in $Tbr1^+$ neurons, increased dendritic spines and microglia, as well as developmental defects in oligodendrocytes and neocortical myelination in the PFC (Usui et al., 2021). Researchers also found the *zbtb16* KO mice exhibited abnormal behaviours such as social impairment and repetitive behaviours, which are characteristic symptoms of autism spectrum disorder. Furthermore, the mice displayed social and cognitive impairment as well as risk-taking behaviours which are relevant to schizophrenia (Usui et al., 2021).

5.2. The Protective Role of PLZF in the CNS

5.2.1. PLZF in Acoustic Trauma

Exposure to very loud sounds can produce permanent hearing loss or acoustic trauma by severely damaging the sensory cells and auditory neurons in the cochlea (Peppi et al., 2011). Yet, the cochlea can be conditioned to withstand damage by prior exposure to moderate-level sounds. It has been shown that PLZF is responsive to corticosteroids (Fahnenstich et al., 2003; Wasim et al., 2010), expressed in the inner ear (Nagy et al., 2005), and interacts with prestin (Nagy et al., 2005), a cochlear motor protein responsible for amplifying acoustic input to the cochlea (Zheng et al., 2000). Therefore, PLZF has been identified as a corticosteroid-responsive transcription factor due to its role in mediating conditioned protection of the cochlea from acoustic trauma. Heterozygous mutant (*luxoid.*$ZBTB16^{LU}$/J) mice deficient in PLZF have hearing and responses to acoustic trauma similar to the WT, however, they are unable to resist cochlear damage by generating conditioning-induced protection from acoustic trauma (Peppi et al., 2011).

In a study by Peppi and colleagues, mice were exposed to acoustic trauma resulting in cochlear damage, then subject to conditioning stimuli including restraint stress, dexamethasone

administration and moderate to high level acoustic stimulation (Peppi et al., 2011). Results showed that following conditioning stimuli, PLZF mRNA levels in the cochlea were increased and the PLZF immunoreactivity was present in the spiral ganglion, lateral wall of the cochlea, organ of Corti and the brain (Peppi et al., 2011). These findings demonstrate that PLZF plays a role in the protective mechanisms in acoustic trauma and may be a potential target for the treatment of other forms of hearing loss or brain injury.

5.2.2. PLZF in Stroke

Stroke is a major contributor to disability and is the second leading cause of death worldwide, with ischemic stroke being the most common type (Kuriakose & Xiao, 2020). Animal studies show that blockade of the angiotensin II type 1 receptor (AT_1R) ameliorates neurological outcome after cerebral ischemia, and these protective effects are partially mediated by AT_2R (Seidel et al., 2011; Krikov et al., 2008). PLZF was identified as a direct adapter protein of the AT_2R and human RER, and both receptors constitute the RAS, which is mainly known for its important role in cardiovascular pathophysiology as well as its crucial functions in the CNS. This suggests that PLZF may be involved in neuroprotection, since it is expressed in temporally dynamic and spatially restricted patterns during brain development (Cook et al., 1995).

Researchers found that PLZF and its receptors AT_2R and RER displayed a ubiquitous expression pattern in various brain regions, including the cortex and striatum (Seidel et al., 2011). Moreover, PLZF overexpression in human neuronal cells was shown to mediate neuroprotection in the glutamate toxicity model *in vitro* (Seidel et al., 2011). Whereas in the *in vivo* stroke model, PLZF mRNA and protein were downregulated on the ipsilateral side and the

neurodetrimental PLZF target genes cyclin A2 and pro-apoptotic factor BID were upregulated (Seidel et al., 2011). Lastly, results show that the stable overexpression of PLZF caused the upregulation of the neuroprotective AT_2R (Seidel et al., 2011). Thus, these findings show that stroke decreases the ipsilateral expression of PLZF, and PLZF regulates certain neuroprotective genes like AT_2R and neurodetrimental genes like cyclin A2.

5.3. PLZF in Neurodegeneration

5.3.1. PLZF in Neuroinflammation

Neuroinflammation is the activation of the innate immune system of the CNS and is an underlying contributor to neurodegenerative diseases such as AD, PD, multiple sclerosis, and amyotrophic lateral sclerosis (Ji et al., 2014; Schwartz & Baruch, 2014). During a neuroinflammatory response, microglia and astrocytes are activated to produce chemokines, cytokines and toxic factors, which may result in neuronal toxicity and neuronal apoptosis (Allaman et al., 2011; Heneka et al., 2015). Since PLZF is known to be a tumour suppressor gene, its dysregulation or overexpression was found to inhibit proliferation and increase apoptosis in various different cancer cell lines (Wang et al., 2013). In the CNS, various reports show that PLZF overexpression inhibits neurogenesis and interestingly, experimental stroke causes a decrease in PLZF expression causing neuroprotection through regulation of neuroprotective and neurodetrimental factors (Seidel et al., 2011; Sobieszczuk et al., 2010). Therefore, the role of PLZF in regulation of CNS diseases involving neuroinflammation is still limited.

To investigate the role of PLZF in neuroinflammation, He and colleagues developed a neuroinflammatory model by injection of lipopolysaccharide into the lateral ventricle of adult rats and detected increased expression of PLZF in the cortex (Bonow et al., 2009; He et al.,

2016). Results show that PLZF expression was specifically upregulated in neurons and consequently, there was an increase in active caspase-3, cyclin D1 and CDK4 levels in vitro and in vivo (He et al., 2016). Moreover, the expression of these cell-cycle regulatory proteins in cortical primary neurons was inhibited after knocking down PLZF by siRNA (He et al., 2016). Therefore, these findings suggest that the upregulation of PLZF may participate in regulation of neuronal apoptosis in neuroinflammation.

5.3.2. PLZF in Autophagy

Autophagy is a fundamental intracellular catabolic mechanism involved in the removal of misfolded proteins. If left to accumulate, these misfolded proteins can damage cells. The onset of a majority of neurodegenerative diseases, such as AD, HD and PD are caused by the build-up of misfolded proteins in the brain (Watanabe, Taguchi, & Tanak, 2020). The process of autophagy involves the formation of autophagosomes which is tightly regulated by an adapter protein called autophagy related 14 (Atg14) (Zhang et al., 2015). It is however unclear how cells respond to external signals to regulate the amount of Atg14 and control the levels of autophagy.

Various studies have investigated the role of GPCRs in activation of PLZF which leads to the degradation of Atg14 and inhibition of autophagy (Zhang et al., 2015). Since PLZF has been shown to mediate the binding of Cul-3, a core component of the regulator of cullins-1 (ROC1) E3 ubiquitin protein ligase complex, researchers found that Cul-3-PLZF regulates autophagy by mediating the proteasomal degradation of Atg14 (Mathew et al., 2012) (Zhang et al., 2015). Ultimately, this mechanism can be controlled by GPCR ligands through glycogen synthase kinase 3 beta (GSK3ß) phosphorylation (Zhang et al., 2015). These findings demonstrate that the

inhibition of GPCRs by pharmacological agents leads to the activation of autophagy in the CNS and improves the neuronal dysfunction in AD, HD and PD.

5.3.3. PLZF in Alzheimer's Disease and Huntington's Disease

AD is the most prevalent neurodegenerative disease and is characterized by neurotoxicity mediated by the accumulation of beta amyloid oligomers, resulting in neuronal loss and progressive cognitive decline. The metabotropic glutamate receptor 5 (mGluR5) is known to regulate autophagic machinery via the mTOR/unc-51 like autophagy activating kinase 1 (ULK1) and GSK3β/PLZF pathways in both AD and HD mouse models (Abd-Elrahman et al., 2017; Abd-Elrahman et al., 2018; Abd-Elrahman et al., 2019). Research has shown that pharmacological inhibition of the mGluR5, a member of the Gαq/11 protein receptor family, leads to the reduction of Aβ aggregation and rescues cognitive function in the mouse model of AD. Therefore, increased PLZF expression and reduced ULK1 activity are characteristic properties of AD and HD pathology. Rescuing autophagy can therefore occur through inactivation of mGluR5 which facilitates the loss of PLZF expression and activates ULK1 as a result of ULK-Ser757 dephosphorylation (Abd-Elrahman et al., 2017; Abd-Elrahman et al., 2018; Abd-Elrahman et al., 2019).

Furthermore, another study investigated the role of optineurin (OPTN), a multifunctional protein that governs cellular processes including autophagy, in mediating mGluR5 downstream signaling (Ibrahim et al., 2021). The findings reveal that OPTN is required for mGluR5-activated Ca^{2+} flux and ERK1/2 signaling as well as deletion of OPTN impairs both mTOR/ ULK1 autophagic signaling and GSK3β/PLZF pathways (Ibrahim et al., 2021). Most importantly, in contrast to the findings from AT2R studies, the link between mGluR5 and the downstream regulation of PLZF is not a result of the direct interaction between mGluR5 and PLZF. Thus,

PLZF is a core component of downstream GPCR signaling and plays a major role in regulation of autophagy, which allows PLZF to be a key therapeutic target in the treatment of neurodegenerative disorders.

6. Objectives and Hypothesis

As discussed above, GPCRs govern major physiological processes and are closely regulated by protein-protein interactions, phosphorylation and internalization (Claing et al., 2002; Kohout & Lefkowitz, 2003). Nevertheless, it remains unclear how these cellular processes control the D_1R-class responsiveness in various neurodegenerative conditions. Specifically, the D_1R-class are known to be essential in regulating the reward system, locomotion, memory and learning. Dysfunction in the D_1R-class signaling is implicated in several diseases affecting motor and cognitive functions such as PD, LID, HD, and drug addiction (Beaulieu & Gainetdinov, 2011; Feyder et al., 2011; Klein et al., 2019; Murer & Moratalla, 2011). In the striatum, the D_1R excites SPNs of the direct striatonigral pathway to induce movement and the D_2R inhibits SPNs of the indirect striatopallidal pathway to inhibit movement (Gerfen et al., 1990; Gerfen & Surmeier, 2011). Whereas the striatal D_5R is expressed in the cholinergic interneurons that regulate the SPN activity output (Gerfen et al., 1990; Gerfen & Surmeier, 2011). The hallmark of LID pathophysiology is an abnormal increase in D_1R-G protein coupling and cAMP production in striatal SPNs (Gerfen & Surmeier, 2011; Surmeier et al., 2007). Additionally, in HD and drug addiction, D_1R activation increases NMDA response and promotes neuronal death (Klein et al., 2019). These adverse circumstances could be due to an imbalance of the striatal levels of PKAs, PKCs, GRKs, and arrestins. In animal studies of LID, researchers found an increase of D_1R expression at the cell surface, indicating defects in the internalization of D_1R (Dumartin et al., 2000; Muriel et al., 1999). Moreover, striatal synaptic plasticity is markedly altered in LID and is regulated by the D_5R (Centonze et al., 2003).

Therefore, to better conceptualize and understand the protein-protein interactions implicated in the assembly and regulation of D_1R-class, our lab performed yeast two-hybrid

screening of a human adult brain cDNA library to identify potential interactors with the D_1R and D_5R using the IL3 and CT regions as baits. Using this screen, our lab identified the PLZF protein as a binding partner which interacts in a subtype-specific manner with the IL3 of D_5R and CT of D_1R. Thus, understanding how PLZF regulates the function of the D_1R-class is critical for the development of subtype-specific drugs and improving drug target selectivity. Like other DARs, there is a high degree of identity between the D_1R-class subtypes because of the fully conserved binding sites in the TM domains. The functional phenotype differences and most divergent regions between the D_1R-class are the IL3 and CT (Jackson et al., 2000; Tumova et al., 2003). Therefore, the focus of my M.Sc. project is to develop an understanding of the detailed mechanisms governing the role of PLZF in regulation of the D_1R and D_5R through binding to the CT and IL3, respectively.

I hypothesize that PLZF interacts with the D_1R-class through complex formation, modulation of D_1R-class signaling cascades and trafficking mechanisms.

I propose these objectives to test my hypothesis:

- Characterize the interaction between D_1R-class and PLZF
- Analyze the role of PLZF in modulation of the D_1R-class pharmacological (ligand binding) properties
- Analyze the role of PLZF as a novel signaling regulator of D_1R-class - cAMP signaling pathway
- Analyze the role of PLZF on D_1R-class-mediated ERK1/2 activation
- Assess the role of PLZF in modulation of D_1R-class trafficking through observation of cell surface and total receptor expression, as well as internalization of the receptor following agonist stimulation

MATERIALS AND METHODS

1. Materials

Eagle's minimal essential medium (EMEM), Dulbecco's modified eagle medium (DMEM), incubation buffer (EMEM without phenol red) and phosphate-buffered saline (PBS) are from Wisent Bioproducts (Sainte-Jean-Baptiste, QC, Canada). Trypsin-ethylenediaminetetraacetic acid (EDTA), bovine calf serum (BCS), fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and gentamicin are from Gibco (Burlington, ON, Canada). Biosafe II scintillation cocktail is from Research Products International (Mount Prospect, IL, USA). Bio-Rad protein assay dye reagent concentrate and Triton-X are from Bio-Rad Laboratories (Mississauga, ON, Canada). [N-Methyl-^{3}H]-SCH23390 is from PerkinElmer (Boston, MA, USA). Ascorbic acid (AA), DA, *cis*-flupenthixol, dihydrexidine, thiothixene, aprotinin, soybean trypsin inhibitor, leupeptin, pepstatin A, benzamidine, phenylmethylsulfonyl fluoride (PMSF), D-Luciferin, Bovine serum albumin (BSA) and anti-FLAG M2 affinity gel are from Sigma-Aldrich (Oakville, ON, Canada). Rabbit monoclonal anti-α-tubulin antibody, rabbit monoclonal anti-ERK1/2 antibody and rabbit monoclonal anti-p-ERK1/2 antibody are from New England Biolabs (Whitby, ON, Canada). Rabbit monoclonal anti-PLZF antibody and rabbit monoclonal anti-β-arrestin 2 antibody are from Cell Signaling Technology (Danvers, MA, USA). Monoclonal anti-FLAG M2-peroxidase (HRP) antibody from Millipore Sigma (Oakville, ON, Canada). ECL anti-rabbit IgG, horseradish peroxidase-linked species specific whole Ab from donkey and Amersham ECL prime from GE Healthcare (Mississauga, ON, Canada). Paraformaldehyde is from Electron Microscopy Sciences (Hatfield, PA, USA). O-Phenylenediamine Dihydrochloride (OPD) substrate and stable peroxide substrate buffer are from ThermoFisher Scientific (Burlington, ON, Canada).

2. Methods

2.1. Cell Culture and Transfection

HEK293 cells were cultured between passages 40-52 in EMEM supplemented with 10% (v/v) heat-inactivated FBS and 40 μg/ml gentamicin, and placed in incubators containing a 5% CO_2 atmosphere at 37°C. Cells were seeded in 100mm culture dishes (approximately 2.5 million cells/dish) and cells were transiently transfected with human D_1R or human D_5R, and human HA-PLZF subcloned in the mammalian expression vector pCMV5, using the calcium phosphate transfection method (Plouffe et al., 2010). The total amount of plasmid DNA per dish was 5 μg and cells were transfected with either D_1R or D_5R alone, or with HA-PLZF. For the control or mock condition, cells were transfected with 5 μg of empty pCMV5 vector. After 18 hours, transfected cells were washed with PBS, detached using trypsin, then resuspended in complete EMEM and reseeded in 100 mm dishes for radioligand binding assays and co-immunoprecipitation experiments, 150 mm dishes for saturation and competition studies, 6-well plates for ERK1/2 activation assays and western blot sample preparations, 12-well plates for ELISA experiments and 96-well plates for cAMP Glosensor assays. The cells were grown for an additional 48 hours before performing experiments.

2.2. Radioligand Binding Assay

The radioligand binding assay was performed to assess the D_1R and D_5R expression levels in transfected HEK293 and HEK293T cells by obtaining the total number of D_1R and D_5R binding sites. Cells were then washed with cold PBS, then cold lysis buffer (10 mM Tris-HCl, pH 7.4; 5 mM EDTA, pH 8.0) was added and cells were scraped from the 100 mm culture dishes and transferred into centrifuge tubes to be centrifuged at 18000 rpm at 4°C for a duration of 20

mins. Pellets were then homogenized in cold lysis buffer using the Brinkman Polytron 3000 homogenizer at a speed of 17,000 rpm for 15 secs, then centrifuged again for 20 min at 18000 rpm and 4°C. The resulting membrane pellet was homogenized at 17000 rpm for 20 sec in resuspension buffer (62.5 mM Tris-HCl, pH 7.4; 1.25 mM EDTA, pH 8.0). 100 µl of membrane homogenate was then added to test tubes containing 300 µl of binding buffer (62.5 mM-Tris HCl;. pH 7.4; 1.25 mM EDTA pH 8.0; 200 mM NaCl; 8.33 mM KCl; 6.7 mM $MgCl_2$; and 2.5 mM $CaCl_2$), 50 µl of [N-Methyl-^{3}H]-SCH23390 and 50 µl of either *cis*-flupenthixol or water. Therefore, the 500 µl mixture for the final assay contained: 50mM Tris-HCl, pH 7.4; 1mM EDTA; 120 mM NaCl; 5 mM KCl; 4mM $MgCl_2$; 1.5 mM $CaCl_2$; and a concentration of approximately 7-9 nM of [N-Methyl-^{3}H]-SCH23390, either in the absence or presence of 10 µM *cis*-flupenthixol. This allows the determination of total and non-specific [N-Methyl-^{3}H]-SCH23390 binding to membranes. Membranes were subsequently incubated within the binding reaction mixtures for 90 mins and then was terminated by rapid filtration through glass fiber filters using a Brandel M-48 Semi-Automated Harvesting System. Next, filters were washed three times with cold washing buffer (50 mM Tris-HCl, pH 7.4; and 100 mM NaCl) and added to scintillation vials containing Bio-Safe II biodegradable scintillation fluid. The radioactive [N-Methyl-^{3}H]-SCH23390 bound to the filters were measured using a Beckman LS6500 liquid scintillation counter. The Bradford protein assay was conducted to determine the total membrane protein concentration of each reaction mixture using the Bio-Rad protein assay dye reagent in addition to the remaining homogenized membrane protein with the BSA as a standard. Total membrane protein concentration and [N-Methyl-^{3}H]-SCH23390 radioactivity in dpm were used to calculate B_{max} in pmol/mg membrane proteins.

2.3. Saturation and Competition Binding Studies

For the saturation and competition radioligand binding studies, HEK293 cells were transfected using 4.5 μg of plasmid DNA for maximal expression of D_1R or D_5R, in the absence and presence of 0.5 μg HA-PLZF. Crude membrane preparation was performed from cells reseeded in 150 mm dishes. Cells were washed with cold PBS, scraped in cold lysis buffer (10 mM Tris-HCl, pH 7.4; 5 mM EDTA, pH 8.0) and centrifuged for 20 mins at 18000 rpm and 4°C. Pellets were washed with lysis buffer and homogenized using the Brinkman Polytron at a speed of 17000 rpm for 15 secs, then centrifuged again for 20 mins at 18000 rpm and 4°C. Pellets were homogenized again in lysis buffer. A sample of the homogenized membrane was immediately used for saturation studies or frozen in liquid nitrogen and stored at -80°C to be used for competition studies at a later time. Either fresh or frozen (thawed on ice) membrane samples were diluted in resuspension buffer (62.5 mM Tris-HCl, pH 7.4; 1.25 mM EDTA, pH 8.0). Saturation studies were performed using increasing concentrations of D_1R-class radioligand [N-Methyl-^{3}H]-SCH23390 (~0.02-7 nM) and 100 μl of fresh membrane preparation in binding buffer. The final mixture is composed of 50 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0; 120 mM NaCl; 5 mM KCl; 4 mM $MgCl_2$; 1.5 mM $CaCl_2$) for a total volume of 500 μl. Non-specific binding was assessed in the presence of 10 μM *cis*-flupenthixol. Competition studies were performed using thawed frozen membrane preparations. A fixed concentration of [N-Methyl-^{3}H]-SCH23390 (~0.5 nM) was used, depending on the obtained K_D value measured from the saturation studies, and increasing concentrations of cold ligands were used. DA was dissolved in 0.1 mM AA while dihydrexidine (DHX), thiothixene (THX), and SCH23390 were dissolved in water. Similarly to the previous method, the membranes were incubated within the binding reaction mixtures for 90 mins and then was terminated by rapid filtration and the radioactive [N-

Methyl-^{3}H]-SCH23390 bound to the filters were measured. The Bradford protein assay was conducted to determine the total membrane protein concentration of each reaction mixture.

2.4. cAMP Glosensor Assay

HEK293T cells were transfected with 0.01 μg D_1R or D_5R with and without 0.5μg HA-PLZF. Total amount of transfected DNA was consistently kept at 5 μg per dish and empty vector (pCMV5) was used to reach this amount for each condition. After 18 hours, HEK293T cells stably expressing Glosensor were reseeded (15,000 cells per well) in poly-L-lysine (PLL; 25 μg/ml)-coated 96-well plates through the use of a multichannel pipette. 24 hours later, the media was removed from the 96-well plates and cells were incubated with 0.3 mg/nl luciferin (dissolved in 1X HBSS + 20mM HEPES; pH 7.4) for 15 mins at room temperature. Cells were then stimulated with 12 increasing concentrations of DA (AA treatment for minimal concentration and 100 μM DA for maximal concentration) for 15 mins at room temperature. The luminescence activity was measured using the Synergy H1Multi-Mode Plate Reader. Radioligand binding assays and western blots were performed to obtain expression levels of D_1R or D_5R alone or with HA-PLZF.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

HEK293 cells were transfected with mock (empty pCMV5 vector) and (either 0.01 μg for internalization or 4.5 μg for cell surface and total expression) FLAG-D_1R and FLAG-D_5R in the absence and presence of 0.5 μg HA-PLZF and other DNA plasmids (1 μg β-arrestin 2, 1 μg β-arrestin 2-V54D). Following transfection, cells were reseeded in 12-well plates. For the internalization assay, existing culture media was aspirated and replaced with 2 ml of incubation

buffer in each well, then cells were treated with either DA (10 μM final concentration) or AA as vehicle (100 μM final concentration) to assess DA-induced internalization. Cells were then returned to 37°C, 5% CO_2 environment for 15 mins. For the recycling assay, the 15 min. DA treatment used to assess internalization was aspirated, cells were treated with either AA or DA again, and incubated for different durations (10, 30, 60 and 90 mins). Afterwards, media was aspirated and cells were fixed with PBS containing 3.7% (v/v) paraformaldehyde for 10 mins at room temperature. Following fixation, cells were washed twice with PBS containing 0.2% (w/v) BSA and then incubated with PBS containing 1% (w/v) BSA for 30 mins. Then, media was aspirated and cells were incubated with monoclonal anti-flag M2 antibody conjugated to horseradish peroxidase (HRP) diluted 1:20000 in PBS containing 1% (w/v) BSA for 1 hour. Cells were then washed twice with PBS containing 0.2% (w/v) BSA for 10 mins. For total expression, 1% BSA- and 0.2% BSA containing 1% (v/v) Triton-X-100 detergent were used for washing and blocking steps to detect the total expression of the receptors found either on the cell surface or in the cytosol. Next, cells were treated with 400 μl of OPD (0.5 mg/ml dissolved in stable peroxide substrate buffer), and covered with aluminium foil for 15 mins. The reaction was stopped by adding 100 μl of 3N HCl. The solution in each well was transferred to 96-well plates and the optical density (OD) at 490 nm was read using a SpectraMaxM5. The background reading obtained from mock (PCMV5)-transfected cells is subtracted to obtain corrected values. To determine receptor internalization by ELISA, detection of an extracellular N-terminal epitope tag on the receptor is analyzed and this epitope is no longer recognized by the cognate antibody once the receptor is internalized. Background is subtracted using the reading obtained from mock-transfected cells. The percentage of receptor endocytosis is calculated from the OD value of vehicle- and agonist-treated cells [(vehicle-treated – agonist-treated)/(vehicle-treated)].

Radioligand binding assays and western blots were performed to assess expression levels of D_1R or D_5R alone or with HA-PLZF. Western blots were also used to determine expression of β-arrestin 2 and β-arrestin 2-V54D.

2.6. ERK1/2 Activation Assays

HEK293 cells were transfected with 0.01 μg D_1R or D_5R with and without 0.5 μg HA-PLZF. Cells are seeded in 6-well culture plates and on experiment day, existing culture media was replaced with 2 ml of incubation buffer in each well – to be used for AA and DA incubation. Cold 10 mM AA is prepared in Milli-Q water to prepare cold 1mM DA and prevent DA oxidation during the assay. Media was aspirated and 2 ml of incubation buffer was added, then 100 μM final concentration AA or 10 μM final concentration DA was added to wells, and cells were returned to the 37°C, 5% CO_2 environment for different durations (AA treatment for 1 min – indicated as 0 min DA in figures – and DA treatment for 1 min, 2.5 mins, 5 mins, 10 mins, 15 mins, 30 mins, and 60 mins. Following AA and DA treatment, cell lysates were collected and sonicated and lysate protein concentrations were determined to prepare western blot samples.

2.7. Co-Immunoprecipitation

HEK293 cells were transfected with 0.01 μg FLAG- D_1R or FLAG-D_5R with and without 0.5 μg HA-PLZF and/or with 1 μg β-arrestin 2. Empty vector (pCMV5) was added to normalize the total amount of DNA to 5 μg within a 100 mm dish. The following day, cells were then reseeded in 100 mm dishes for co-IP and binding studies. After 48 hours, media was aspirated and cells were incubated in incubation buffer (EMEM without phenol red; 20 mM HEPEs; 10 μg/ml gentamicin) with either DA (10 μM final, dissolved in AA) or AA as a vehicle (0.1 mM

final) for 15 mins at 37°C, 5% CO_2 environment. At the end of incubation, cells were placed on ice, media was aspirated and cells were washed twice with cold PBS. Next, cells were collected in 0.8 ml of cold $RIPA^+$ buffer with protease inhibitors, and lysates were solubilized using end-over-end rotation for 1 hour at 4°C. The solubilized extracts were centrifuged at 12 500 rpm for 15 mins at 4°C. Protein concentration of lysates was measured using Bradford protein assays and BSA was used as a protein standard. 500 µg lysates were incubated with 50 µl of anti-Flag M2 affinity gel for 2 hours at 4°C using the rotating wheel. After rotation, the antibody matrix was washed five times with cold $RIPA^+$ buffer, followed by eluting samples with 60 µl of SDS-PAGE loading buffer overnight at room temperature. According to the measured protein concentrations, 20 µg of protein were aliquoted as input samples and SDS-PAGE loading buffer was added to each sample. D_1R and D_5R expression in the absence and presence of HA-PLZF was determined using radioligand binding assays.

2.8. Western Blot

For each experiment, western blot samples were collected and each sample containing 20 µg of protein were loaded into 10% (v/v) acrylamide gels for SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes at 15 V for 20 mins using Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell. To determine PLZF expression, membranes were cut below 75 kDa marker into two pieces and incubated with blocking buffer (50 mM Tris-HCl, pH 8.0; 80 mM NaCl; 2mM $CaCl_2$; 5% (w/v) dry skim milk; 0.2% (v/v) NP-40 Alternative; and 0.02% (w/v) NaN_3) on a rocking platform shaker at 4°C overnight. The following day, membranes were rinsed with Tris-buffered saline containing Tween 20 (TBS-T) (20 mM Tris-HCl, pH 7.4; 137 mM NaCl; and 0.2% (v/v) Tween 20). Top membrane was

incubated with rabbit monoclonal anti-PLZF antibody (Cell Signaling Technology) diluted 1:2000 in TBS-T and bottom membrane was incubated with monoclonal anti-α-tubulin antibody (New England Biolabs) diluted 1:3000 in TBS-T to assess total protein levels. To determine ERK1/2 and p-ERK1/2 expression, membranes were incubated with monoclonal rabbit anti-ERK1/2 antibody (New England Biolabs) diluted 1:1000 in TBS-T and monoclonal rabbit anti-p-ERK1/2 antibody (New England Biolabs) diluted 1:1000 in TBS-T, respectively. To determine β-arrestin 2 expression, membranes were incubated with monoclonal rabbit anti-β-arrestin 2 antibody (Cell Signaling Technology) diluted 1:2000 in TBS-T. The following day, using a rocking platform shaker and at room temperature, membranes were washed three times with TBS-T for 10 mins, incubated for 1 hour with the ECL anti-rabbit IgG, horseradish peroxidase-linked species specific whole Ab from donkey (GE Healthcare) diluted 1:10000 in TBS-T, then washed four times with TBS-T for 40 mins and protein bands were visualized using Amersham ECL reagents. Images were captured using DNR Bio-Imaging Systems MicroChemi 2.0 gel imaging system and DNR Bio-Imaging Systems GelCapture Image Acquisition software. The densitometric analysis of protein bands was performed using DNR Bio-Imaging Systems GelQuant image analysis software. For the ERK assay, to account for any differences in the level of total protein factoring in to differences in quantified p-ERK1/2, a normalized p-ERK1/2 value was calculated for each lane using the formula (p–ERK1+p–ERK2)/(ERK1+ERK2).

2.9. Statistical Analysis

GraphPad Prism 9.0 was used for all statistical analyses and curve fitting. All data are expressed as mean ± SEM. Binding isotherm analysis using a non-linear regression curve fitting was used to determine the maximal binding capacity (B_{max}, pmol/mg protein) of [N-Methyl-^{3}H]-

SCH23390 for saturation and radioligand binding studies, the equilibrium dissociation constant (K_D, nM) for saturation studies, and the inhibitory dissociation constant of cold drugs (K_I nM) for competition studies. For cAMP Glosensor assay, the raw data for individual dose-response curves of DA or DHX were analyzed to obtain the DA or DHX half maximal effective concentration (EC_{50} or potency) and DA- or DHX-induced maximal activation of AC (E_{max}). The raw data was then normalized as a percent of the best-fitted E_{max} obtained from the receptor alone (D_1R or D_5R). Normalized data was averaged and the curves were analyzed again using a simultaneous fitting with a shared slope parameter. The GraphPad generated best-fitted EC_{50} of DA and DHX were reported. Using GraphPad Prism, different statistical tests were performed for specific results and will be noted in the results section under each experiment, with $p \leq 0.05$ deemed the threshold for statistical significance.

RESULTS

Part 1: Assessing PLZF Interaction with D_1R-Class

1.1. PLZF Interacts with D_1R-Class in Transfected HEK293 Cells

Previous studies have identified PLZF as a binding partner for various proteins and receptors and the preliminary studies from our lab using yeast two-hybrid screening revealed that PLZF interacts with the D_1R at the CT and the D_5R at the IL3. To further validate this interaction, co-IP and immunoblotting (IB) experiments were performed in transfected HEK293 cells under different treatments. In all co-IP experiments, anti-Flag M_2 agarose beads were used to immunoprecipitate the Flag-tagged D_1R (FD_1R) and Flag-tagged D_5R (FD_5R) and the cognate HA-PLZF was analyzed by probing with anti-PLZF antibody in the immunoblots. Primarily, one proof-of-principle experiment was performed to demonstrate the constitutive interaction between PLZF and D_1R as well as PLZF and D_5R. The results from this experiment confirm an interaction between PLZF and D_1R **(Fig. 13 A, B),** and PLZF and D_5R **(Fig. 13 C, D)**.

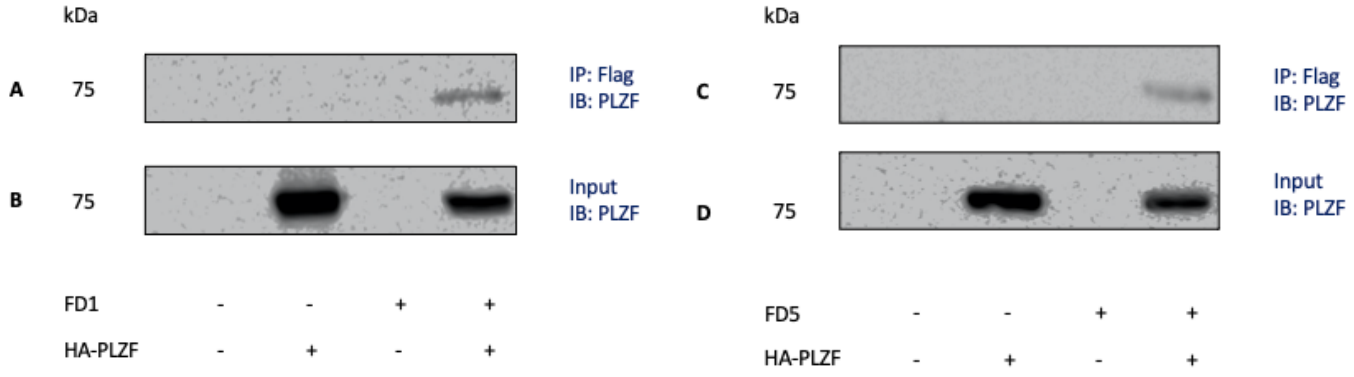


Figure 13. Complex formation between Flag-tagged human D1R/Flag-tagged human D5R and HA-tagged PLZF.
Proof -of-principle experiment of co-immunoprecipitation and immunoblotting of HEK293 cells transfected with FD1 or FD5 with HA-PLZF**. A and C)** FD1 and FD5 were immunoprecipitated using anti-Flag conjugated beads and HA-PLZF was probed using anti-PLZF antibody. **B and D)** Inputs were probed with anti-PLZF antibody. N=1.

1.2. DA and *Cis*-Flupentixol Differentially Modulate D_1R-Class and PLZF Interaction

It has been shown that the stability of the interaction between GPCRs and interacting proteins could be modulated by the presence of agonists or antagonists (Slater et al., 2016; Yarur et al., 2020). Thus, co-IP was performed to test whether the interaction between the D_1R-class and PLZF is altered in the presence of DA. HEK293 cells were co-transfected with either FD_1R±HA-PLZF or FD_5R±HA-PLZF, and cells were incubated with either vehicle (AA) or 10 µM DA for 15 minutes. Results reveal that incubation of cells in the presence of DA significantly increased the amount of PLZF interacting with D_1R and even more so for D_5R **(Fig. 14, 15)**.

In addition, the interaction between the D_1R-class and PLZF was assessed in transfected HEK293 cells using *cis*-flupentixol (flu); a nonselective DAR antagonist that acts as an inverse agonist for D_1R-class. *Cis*-flupentixol reduces constitutive activity of D_1R and D_5R by reverting the conformation of the receptors from an active (R^*) to an inactive conformation (R) (Tiberi & Caron, 1994). Therefore, HEK293 cells were co-transfected with either Flag-D_1R±HA-PLZF or Flag-D_5R±HA-PLZF, and cells were incubated with either 10 µM flu, vehicle (AA) or 10 µM DA for 15 minutes. Treatment of cells using flu resulted in a significant decrease of PLZF recruitment to the D_1R and even further dissociation of the D_5R and PLZF complex **(Fig. 16, 17)**. Whereas, in accordance with the previous results **(Fig. 15)**, DA lead to a stronger interaction between D_1R/D_5R and PLZF **(Fig. 17)**. These results thus indicate that D_1R/ D_5R and PLZF complex formation does not require a ligand, however the presence of an agonist like DA results in further recruitment of PLZF to the receptors and an antagonist like flu abolishes the interaction between the D_1R-class subtypes and PLZF.

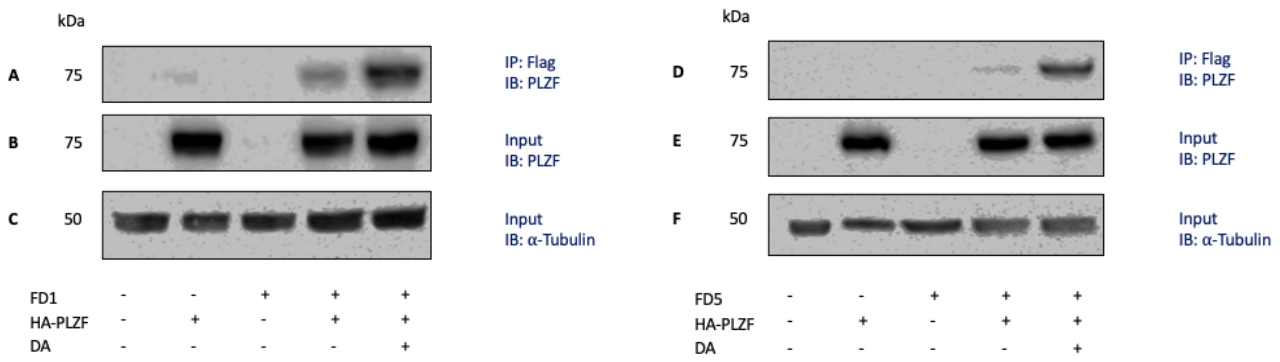


Figure 14. Complex formation between FD1R/FD5R and HA-PLZF under basal and DA stimulation.
Representative experiment of co-immunoprecipitation and immunoblotting of HEK293 cells transfected with FD1 or FD5 with HA-PLZF then subject to 15 mins stimulation with vehicle or DA. **A and D)** FD1 and FD5 were immunoprecipitated using anti-Flag conjugated beads and HA-PLZF was probed using anti-PLZF antibody. **B and E)** Inputs were probed using anti-PLZF antibody. **C and F)** Alpha-tubulin inputs were probed with anti-alpha tubulin antibody. N=6.

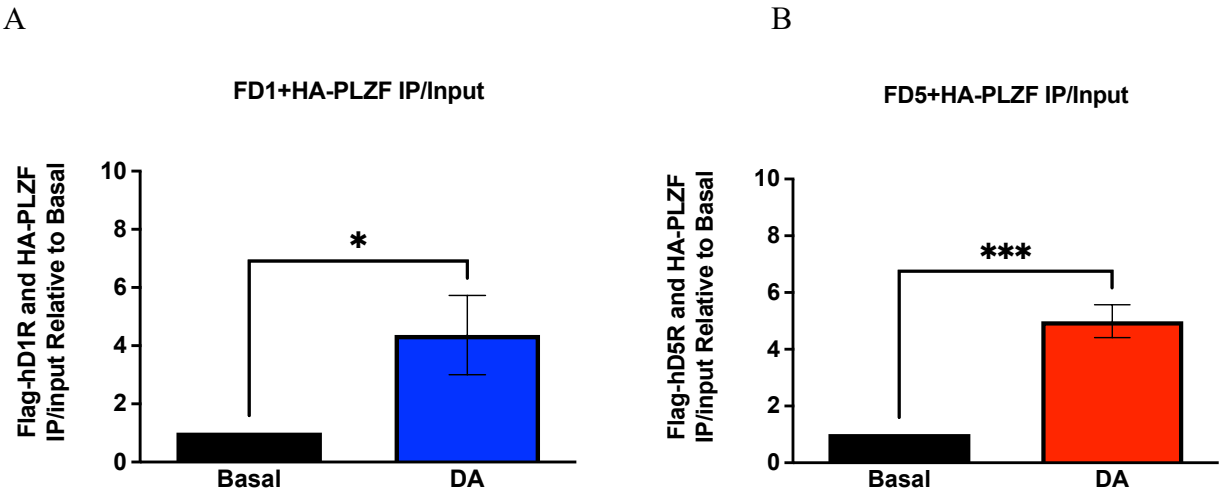


Figure 15. Quantification of the effect of DA stimulation on complex formation between FD1R/FD5R and HA-PLZF.
Densitometric analysis using GelQuant software for the co-immunoprecipitation of **(A)** FD1 with HA-PLZF and **(B)** FD5 with HA-PLZF under stimulation of HEK293 cells using 10 µM DA for 15 mins, relative to basal. Statistical analysis was performed using column analysis and one sample t-test compared to a value of 1. Bars represent mean ± SEM. N=6. Bmax values (mean ± SEM) for FD1, FD1+HA-PLZF, FD5, and FD5+HA-PLZF were 2.13 ± 0.72, 1.91 ± 0.63, 2.05 ± 0.51, and 2.02 ± 0.47.

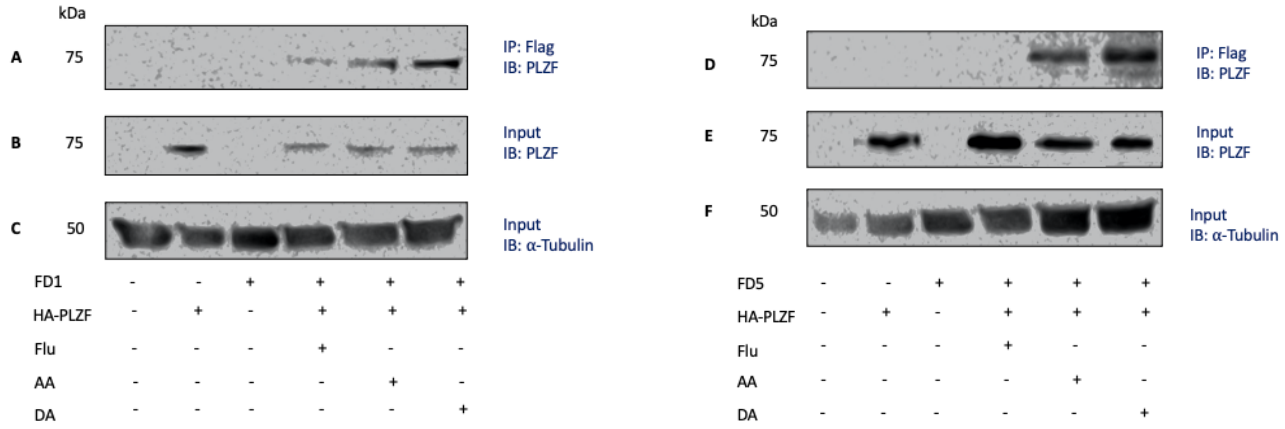


Figure 16. Complex formation between FD1/FD5 and HA-PLZF under basal, flupentixol and DA stimulation.
Representative experiment of co-immunoprecipitation and immunoblotting of HEK293 cells transfected with FD1 or FD5 with HA-PLZF then subject to 15 mins stimulation with vehicle, flu or DA. **A and D)** FD1 and FD5 were immunoprecipitated using anti-Flag conjugated beads and HA-PLZF was probed using anti-PLZF antibody. **B and E)** Inputs were probed using anti-PLZF antibody. **C and F)** Alpha-tubulin inputs were probed with anti-alpha tubulin antibody. N=3

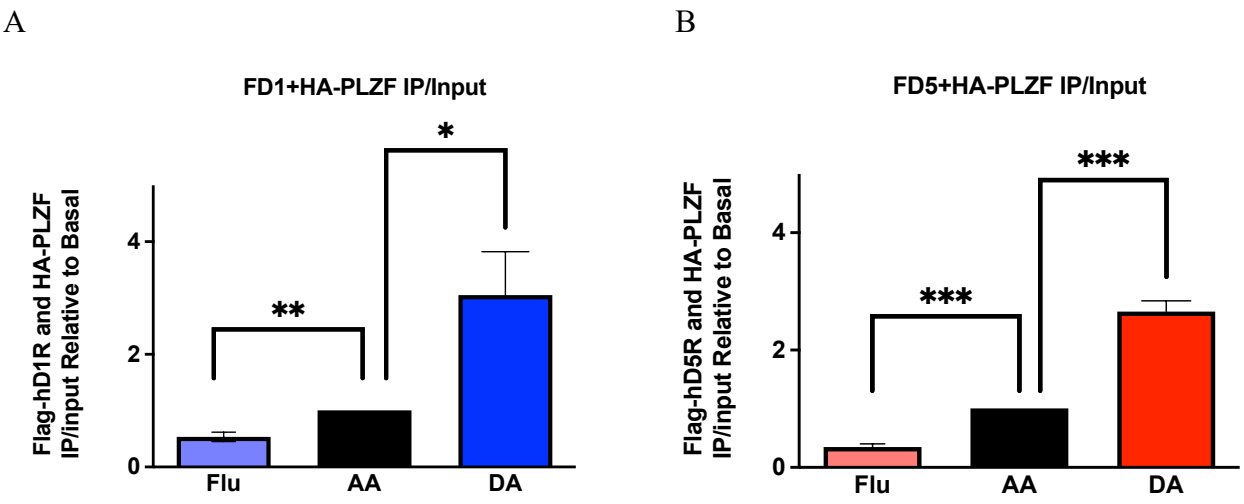


Figure 17. Quantification of the effect of flupentixol and DA stimulation on complex formation between FD1/FD5 and HA-PLZF.
Densitometric analysis using GelQuant software for the co-immunoprecipitation of **(A)** FD1 with HA-PLZF and **(B)** FD5 with HA-PLZF under stimulation of HEK293 cells using 10 µM Flu and 10 µM DA for 15 mins, relative to basal. Statistical analysis was performed using column analysis and one sample t-test and compared to a value of 1. Bars represent mean ± SEM. N=3. Bmax values (mean ± SEM) for FD1, FD1+HA-PLZF, FD5, and FD5+HA-PLZF were 2.65 ± 0.53, 2.03 ± 0.72, 2.33 ± 0.34, and 2.12 ± 0.54.

1.3. PLZF Does Not Alter the Pharmacological Properties of D_1R-Class in HEK293 Cells

Our lab's yeast two-hybrid screen results have shown that PLZF interacts with the CT of D_1R and the IL3 of D_5R. It is known that the CT and IL3 domains of the D_1R-class are critical in modulating the ligand binding properties of the receptors. Evidently, D_5R displays higher constitutive activity, increased agonist affinity and decreased inverse agonist affinity when compared with D_1R in HEK293 cells (Tiberi and Caron, 1994). Therefore, radioligand binding assays were performed to assess whether PLZF impacts the characteristic ligand affinity and binding properties of D_1R and D_5R. Saturation and competition assays were performed on HEK293 cells transfected with D_1R/D_5R alone or with HA-PLZF.

Saturation studies were performed using the D_1R-class specific radiolabeled ligand [^{3}H]-SCH-23390 to determine the K_D and B_{max} of each receptor subtype. The B_{max} values exhibited no significant changes for the D_1R-class alone compared to D_1R/D_5R co-expressed with PLZF. Likewise, the K_D values were similar with no significant difference between D_1R-class alone or co-expressed with PLZF **(Fig. 18)**. Competition studies were performed to determine the affinity to agonists and antagonists, by obtaining the inhibitory constant (K_I) of the D_1R-class alone or co-expressed with PLZF. K_I values were determined using a fixed concentration of [^{3}H]-SCH-23390 and increasing gradient concentrations of cold agonists DA and DHX, as well as inverse agonist THX and antagonist SCH23390. There were no significant differences in the K_I values obtained for the D_1R-class alone or co-expressed with PLZF **(Fig. 19)**. The K_D and B_{max} values are summarized in Table 1 and the Ki values are summarized in Table 2 for D_1R/D_5R in the absence and presence of PLZF. Results reveal that PLZF does not alter the expression and ligand binding properties of the D_1R-class.

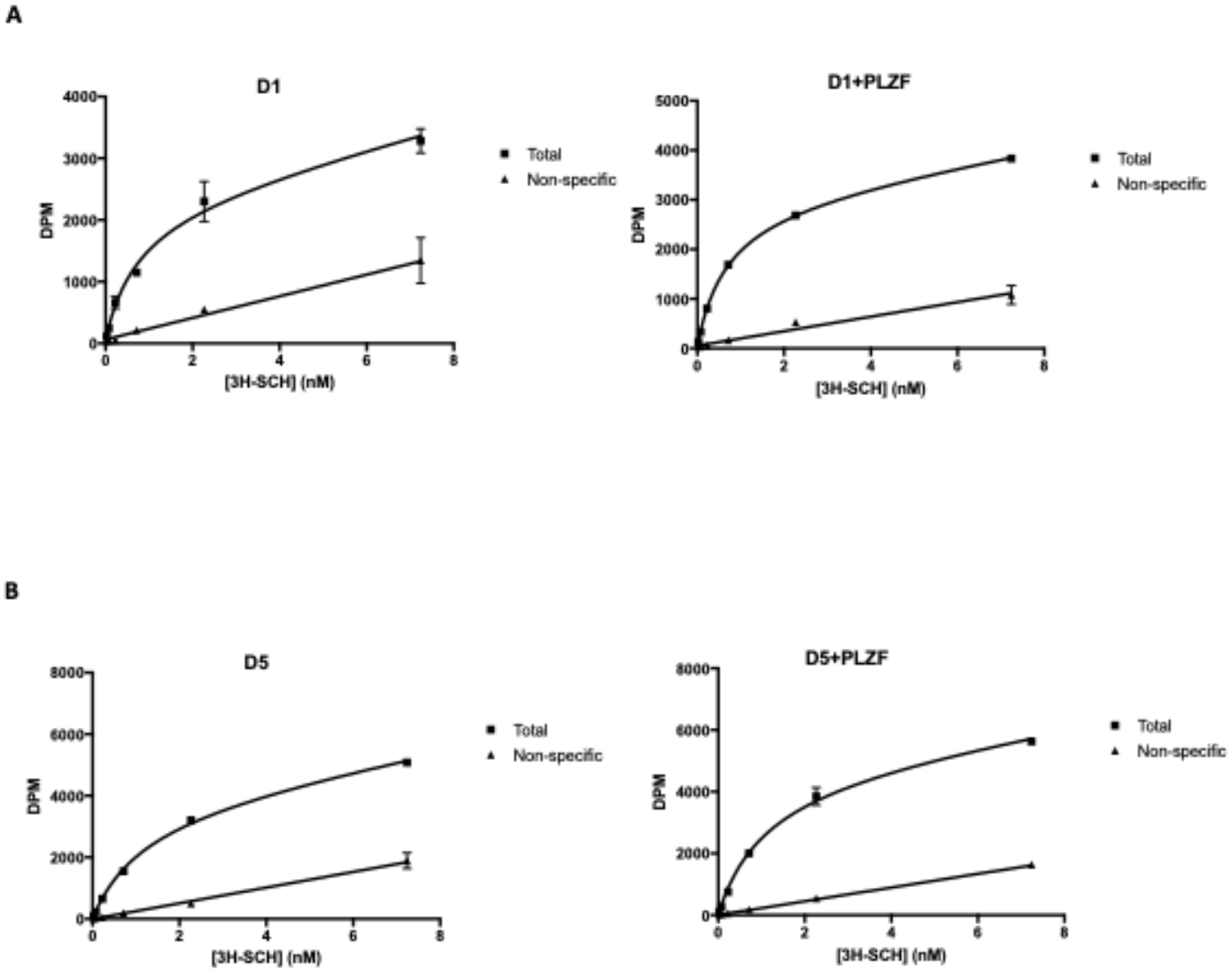


Figure 18. Saturation studies demonstrate ligand binding and expression properties of D1/ D5 in the absence and presence of HA-PLZF.
Representative experiment of saturation radioligand binding assay using [^{3}H]-SCH23390 on HEK293 cells transfected with **(A)** D1 in the absence and presence of HA-PLZF and **(B)** D5 in the absence and presence of HA-PLZF. Curves were generated and analyzed using GraphPad Prism.

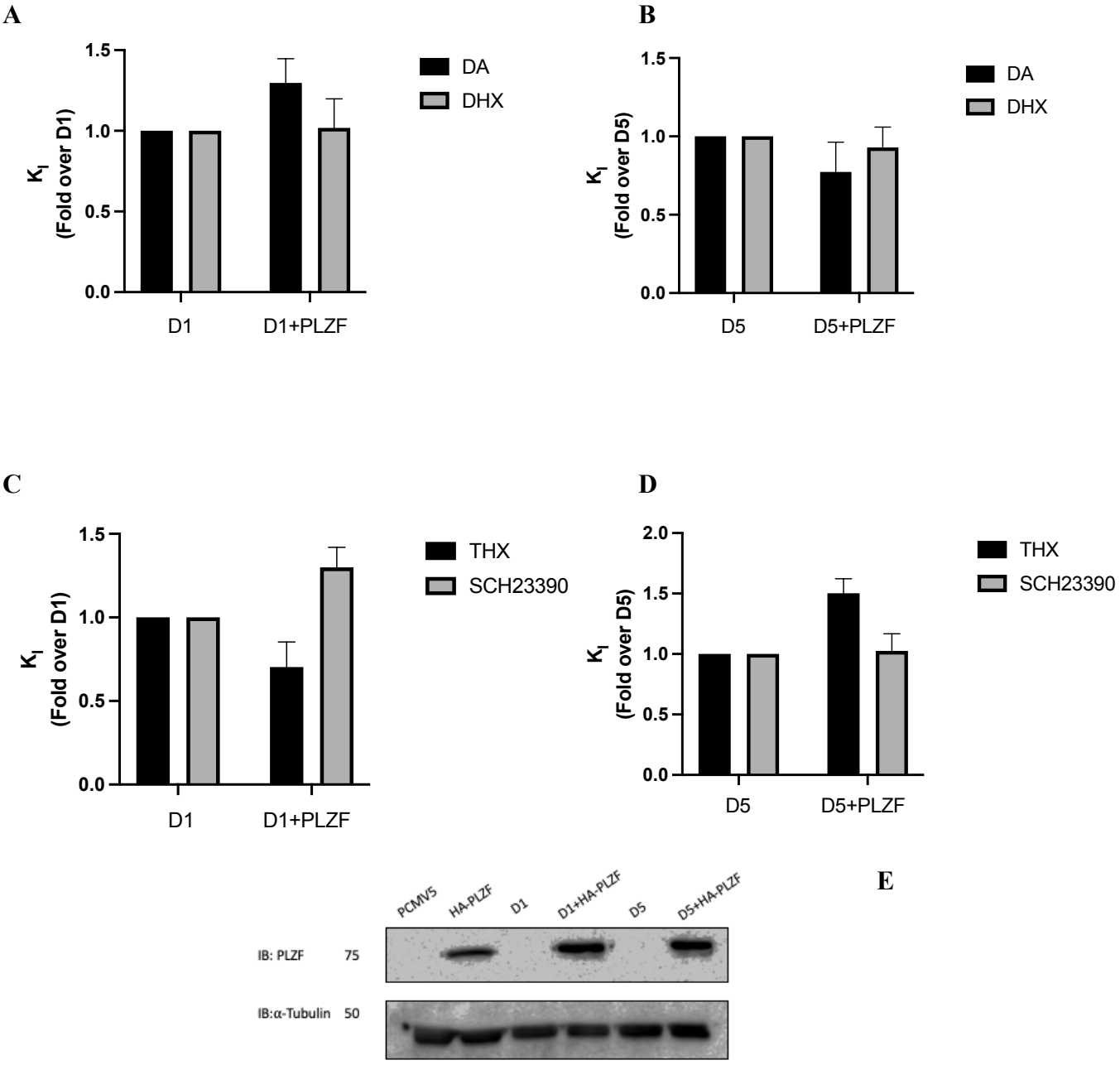


Figure 19. Fold changes value for D1R-class agonists (DA and DHX) and inverse agonist (thiothixene) and antagonist (SCH23390) affinities of D1/D5+PLZF relative to D1/D5. Raw K_I values obtained from competition studies are expressed as fold change relative to the D1/D5 receptor (value set to 1.0). Relative values were compared to 1 using a one-sample t-test (*$p < 0.05$). D1 and D1+PLZF treated with agonists **(A)** and antagonists **(C)**. D5 and D5+PLZF treated with agonists **(B)** and antagonists **(D). (E)** Representative western blot for saturation and competition studies showing HEK293 cells transfected with mock (PCMV5), HA-PLZF, D1, D1+PLZF, D5, D5+PLZF, probed with anti-PLZF antibody and alpha-tubulin antibody.

	Bmax (pmol/mg proteins)	K_D for [^{3}H]-SCH-23390 (nM)
D1 (N=6)	9.978±1.594	0.513±0.065
D1+HA-PLZF (N=6)	8.595±1.554	0.508±0.068
D5 (N=3)	12.397±1.179	1.301±0.105
D5+HA-PLZF (N=3)	14.86±2.823	1.171±0.199

Table 1. Bmax (pmol/mg membrane proteins) and equilibrium dissociation constant (K_D) values for [^{3}H]-SCH-23390 (nM) of HEK293 cells transfected with D1/D5 ± HA-PLZF.
Values are shown as mean ± SEM.

	Dopamine K_I (nM)	Dihydrexidine K_I (nM)	Thiothixene K_I (nM)	SCH23390 K_I (nM)
D1	4250±263	377±48.1	68.48±5.82	0.74±0.11
D1+HA-PLZF	5510±102	384±48.8	48.11±4.12	0.95±0.17
D5	405±6.15	46.9±3.33	144.7±7.83	0.75±0.13
D5+HA-PLZF	313±24.6	43.6±4.83	217.3±7.44	0.77±0.18

Table 2. Inhibitory constant (K_I) values for HEK293 cells transfected with D1/D5 ± HA-PLZF upon exposure to agonists dopamine and dihydrexidine, inverse agonist thiothixene and antagonist SCH23390 (nM).
Values are shown as mean ± SEM. N=3.

Part 2: The Role of PLZF as a Novel Signaling Regulator of D_1R-Class

2.1. PLZF Differentially Modulates the cAMP Response of D_1R-Class in HEK293T Cells

The Glosensor-based cAMP assay was performed to determine the regulation of constitutive and agonist-dependent activity of D_1R and D_5R by PLZF. In this assay, HEK293T cells stably expressing the Glosensor construct are used to allow for cAMP to bind to the cAMP binding domain of PKA-flexible luciferase biosensor eliciting a conformational shift in luciferase that initiates the conversion of D-luciferin substrate to oxyluciferin and the production of light. HEK293T cells were transfected with FD_1R or FD_5R±HA-PLZF, and agonist-dependent coupling to Gs was measured with dose-response curves using DA and the D_1R-class agonist DHX. Since D_1R-class activate AC to increase cAMP production, the Glosensor assay was used to determine whether PLZF plays a role in regulation of the cAMP response in D_1R and D_5R.

Interestingly, when cells are stimulated with DA, PLZF plays a contrasting role in the cAMP response of each receptor by increasing cAMP levels of D_1R and decreasing cAMP levels of D_5R. Specifically, although the EC_{50} for D_1R and D_1R+PLZF is similar (1440 nM and 1498 nM), there is a robust increase (50%) in E_{max} for D_1R+PLZF compared to D_1R **(Fig. 20A)**. When cells expressing D_5R and D_5R+PLZF were stimulated with DA, PLZF results in a 30% decrease in the E_{max} in comparison to D_5R alone **(Fig. 20A).** A similar trend was observed when cells were stimulated with DHX **(Fig. 20B),** validating the effect that was shown with DA stimulation. Therefore, PLZF expression causes an opposite effect on the DA and DHX-mediated maximal activation of D_1R and D_5R, but has minimal effect on agonist potency.

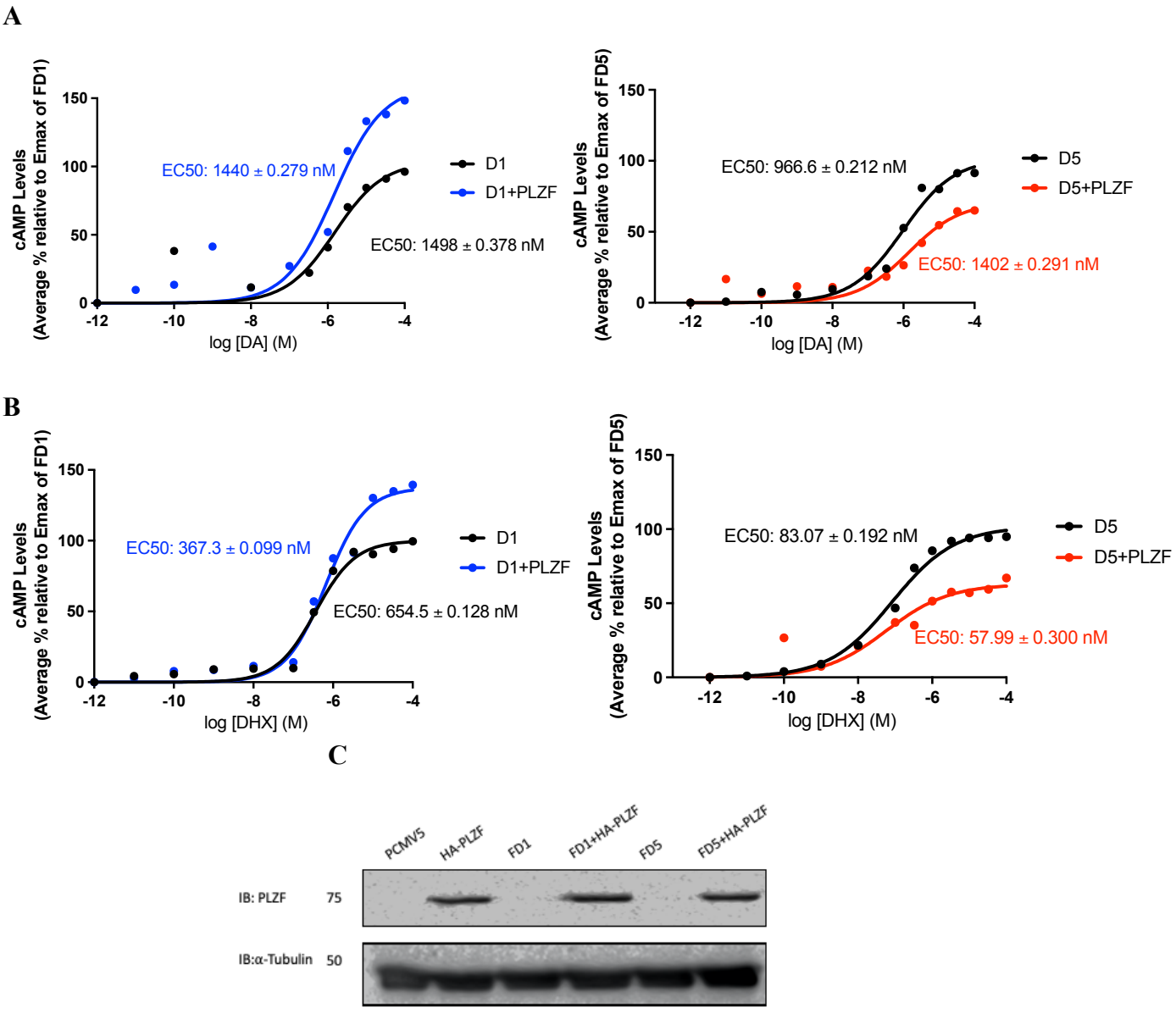


Figure 20. Glosensor-based cAMP assay using agonists DA and DHX on HEK293T cells transfected with FD1/FD5 + HA-PLZF.

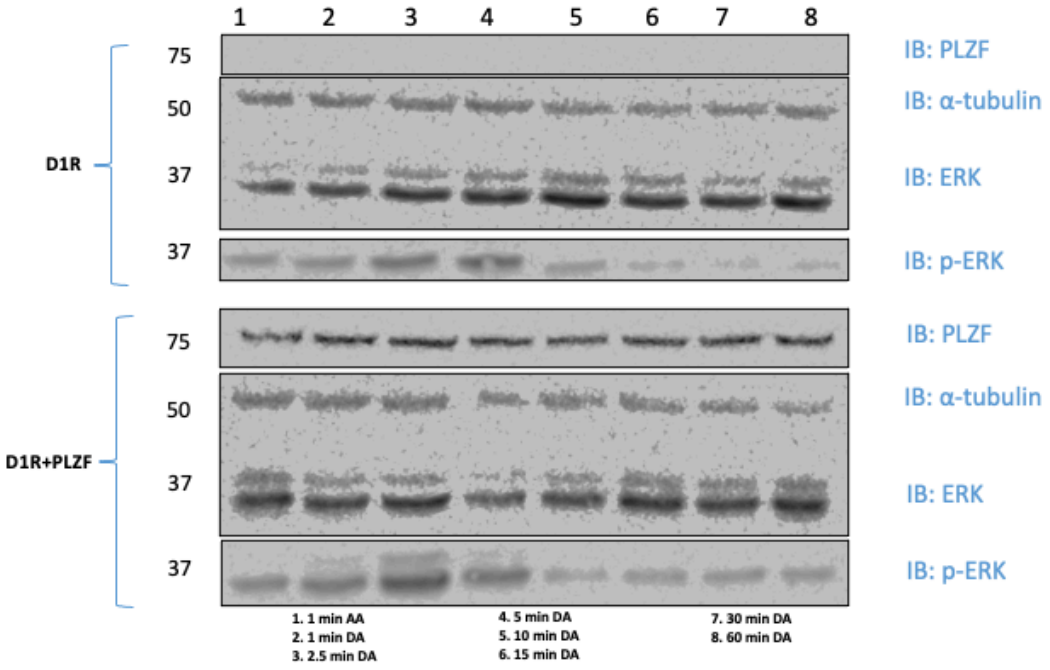
Representative experiment of cAMP dose-response curves using agonists dopamine (DA) and dihydrexidine (DHX). Curves were generated using GraphPad Prism. Averaged EC50 values and standard errors are shown on each graph. Intracellular cAMP values are represented on the y-axis by cAMP levels (% relative to Emax of FD1/FD5 alone). **A)** HEK293T cells transfected with FD1/FD5 + HA-PLZF are stimulated with increasing doses of DA to obtain Emax and EC50 values. N= 4. **B)** HEK293T cells transfected with FD1/FD5 + HA-PLZF are stimulated with increasing doses of DHX to obtain Emax and EC50 values. N=3. **C)** Representative blot for glosensor-based cAMP assay showing HEK293T cells transfected with mock (PCMV5), HA-PLZF, FD1, FD1+PLZF, FD5, FD5+PLZF, probed with anti-PLZF antibody and alpha-tubulin antibody. B_{max} values in pmol/mg proteins (mean ± SEM) for FD1, FD1+HA-PLZF, FD5, and FD5+HA-PLZF were 2.341 ± 0.81, 2.14 ± 0.52, 2.075 ± 0.21, 2.13 ± 0.73.

2.2. PLZF Induces D_1R-mediated ERK1/2 Activation and Reduces D_5R-mediated ERK1/2 Activation in HEK293 Cells

ERK1/2 are downstream mediators of signal transduction from the cell surface receptors to the nucleus and are also known to be activated downstream of Gαs-linked receptors by cAMP. Studies have shown that striatal overactivation of ERK1/2 via D_1R is the hallmark of a hypersensitive molecular response associated with dyskinetic behaviours seen in many neurodegenerative diseases (Fieblinger et al., 2014). Hence, it is important to characterize the role of PLZF in regulation of D_1R/D_5R-mediated ERK1/2 activation to better understand the effect of PLZF on D_1R/D_5R signaling cascades. HEK293 cells were transfected with D_1R or D_5R± HA-PLZF and stimulated with DA for 1, 2.5, 5, 10, 15, 30 and 60 mins, or left unstimulated (AA).

Time course experiments were performed to assess the ability of PLZF to modulate the temporal ERK1/2 activation by D_1R-class. The results suggest that PLZF significantly increases the magnitude of D_1R-mediated ERK1/2 activation at 1, 2.5 and 5 mins timepoints then decreases back to basal D_1R level by 10 mins **(Fig. 21B).** Next, the effect of PLZF on D_5R-mediated ERK1/2 activation was assessed. The results reveal that PLZF significantly inhibits D_5R-mediated ERK1/2 activation at 1 and 2.5 mins, then continues to inhibit at 5 mins until it reaches basal D_5R ERK1/2 levels by 10 mins **(Fig. 21D).** These results confirm the role of PLZF in mediating opposing effects on D_1R vs. D_5R signaling **(Fig. 21B, D)**. The observed trend from this experiment is in line with the findings from the cAMP assay that reveal that PLZF also plays a similar opposing role in cAMP production in D_1R/D_5R. However, these results may be independent of the cAMP results reported earlier given the apparent interaction between PLZF and ERK1/2 seen in the cyclical activity of spermatogonial stem cells (Zhuang et al., 2019).

A


1 2 3 4 5 6 7 8
D1R
D1R+PLZF
75
50
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37
75
50
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37
IB: PLZF
IB: α-tubulin
IB: ERK
IB: p-ERK
IB: PLZF
IB: α-tubulin
IB: ERK
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1. 1 min AA
2. 1 min DA
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6. 15 min DA
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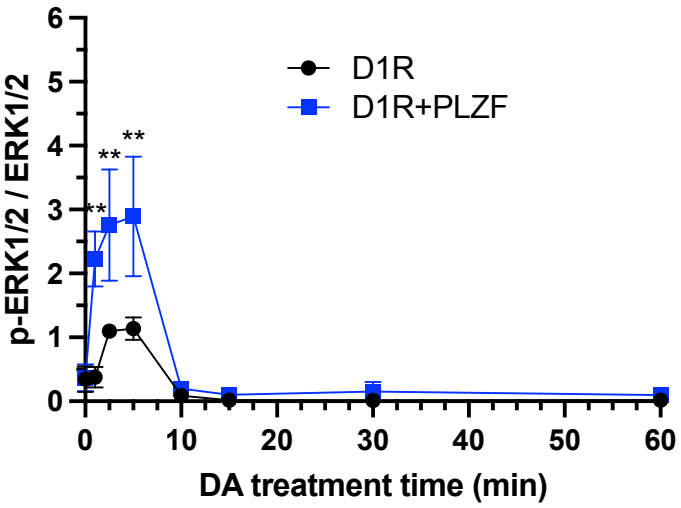
B


D1R
D1R+PLZF
p-ERK1/2 / ERK1/2
DA treatment time (min)
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1
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3
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5
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0
10
20
30
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60
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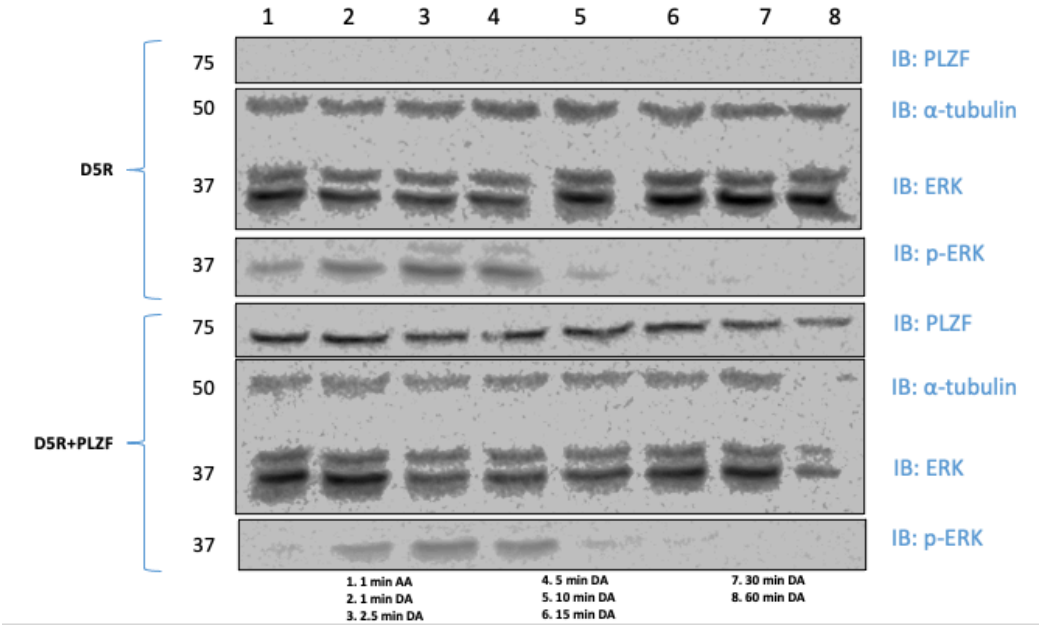
C



D

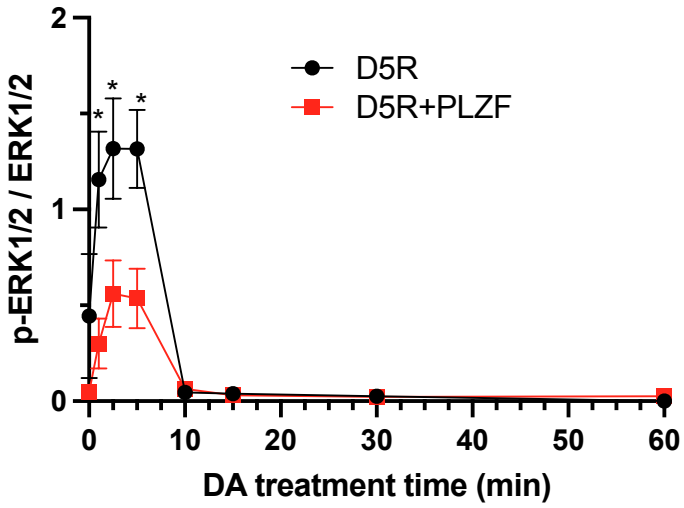


Figure 21. Time course of D1R/D5R-mediated ERK1/2 activation in HEK293 cells
Representative experiments using HEK293 cells transfected with **A)** D1 ± HA-PLZF and **C)** D5 ± HA-PLZF were stimulated with DA for 1, 2.5, 5, 10, 15, 30 and 60 mins, then cell lysates were gathered and subjected to immunoblotting with anti-PLZF, anti-alpha-tubulin, anti-ERK, and anti-phospho-ERK antibodies. Densitometric analysis using GelQuant software reveals increase in ERK activation when PLZF is co-transfected with **(B)** D1R and **(D)** D5R. N=4. Statistical analysis was performed using multiple unpaired t-tests (*$p < 0.05$) and error bars are SEM. B_{max} in pmol/mg proteins for [^{3}H]-SCH23390 expressed as means± SEM for D1R, D1R+HA-PLZF, D5R, D5R+HA-PLZF were 7.065 ± 2.06, 4.183 ± 0.99, 1.71 ± 0.87, 1.148 ± 0.84.

Part 3: The Role of PLZF in Modulation of D_1R-Class Trafficking Properties

3.1. PLZF Increases Cell Surface and Total Expression of D_1R in HEK293 Cells

As shown in studies where PLZF interacts with the GPCR AT_2R, angiotensin II stimulation induces cytosolic PLZF to co-localize with AT_2 at the plasma membrane, then drives AT_2 and PLZF to internalize (Senbonmatsu et al., 2003). Therefore, to assess whether PLZF modulates the cell surface expression and total expression of the D_1R-class receptors, ELISA was performed on HEK293 cells transfected with FD_1R or FD_5R±HA-PLZF. The total receptor expression was obtained by treating cells with Triton-X to permeabilize the cells and detect the receptor located in the cytosol as well as on the cell surface. Corrected OD values for the D_1R/D_5R alone and with PLZF were measured by subtracting the OD reading from mock-transfected cells. Results show that PLZF significantly increases cell surface (~60%) and total expression (~40%) of D_1R but has no significant effect on D_5R **(Fig. 22)**.

3.2. PLZF Impedes D_1R Internalization and Promotes D_5R Internalization in HEK293 Cells

The ELISA was also used to determine whether PLZF modulates the internalization of D D_1R/D_5R. HEK293 cells were transfected with FD1R or FD5R±HA-PLZF and were stimulated with DA for 15 minutes prior to the ELISA. Internalization of the receptor was determined by the use of the HRP-conjugated anti-Flag antibody in the ELISA. Once the flag-tagged receptor internalizes, it will not be recognized by the antibody. Therefore, the percentage of endocytosed receptor is calculated from the OD values obtained for vehicle and agonist-treated cells [(vehicle-treated – agonist treated)]/vehicle treated)]. Results reveal that PLZF facilitates the internalization of the D_5R **(Fig. 23A)** while it impedes the internalization of D_1R, following DA stimulation **(Fig.23A).**

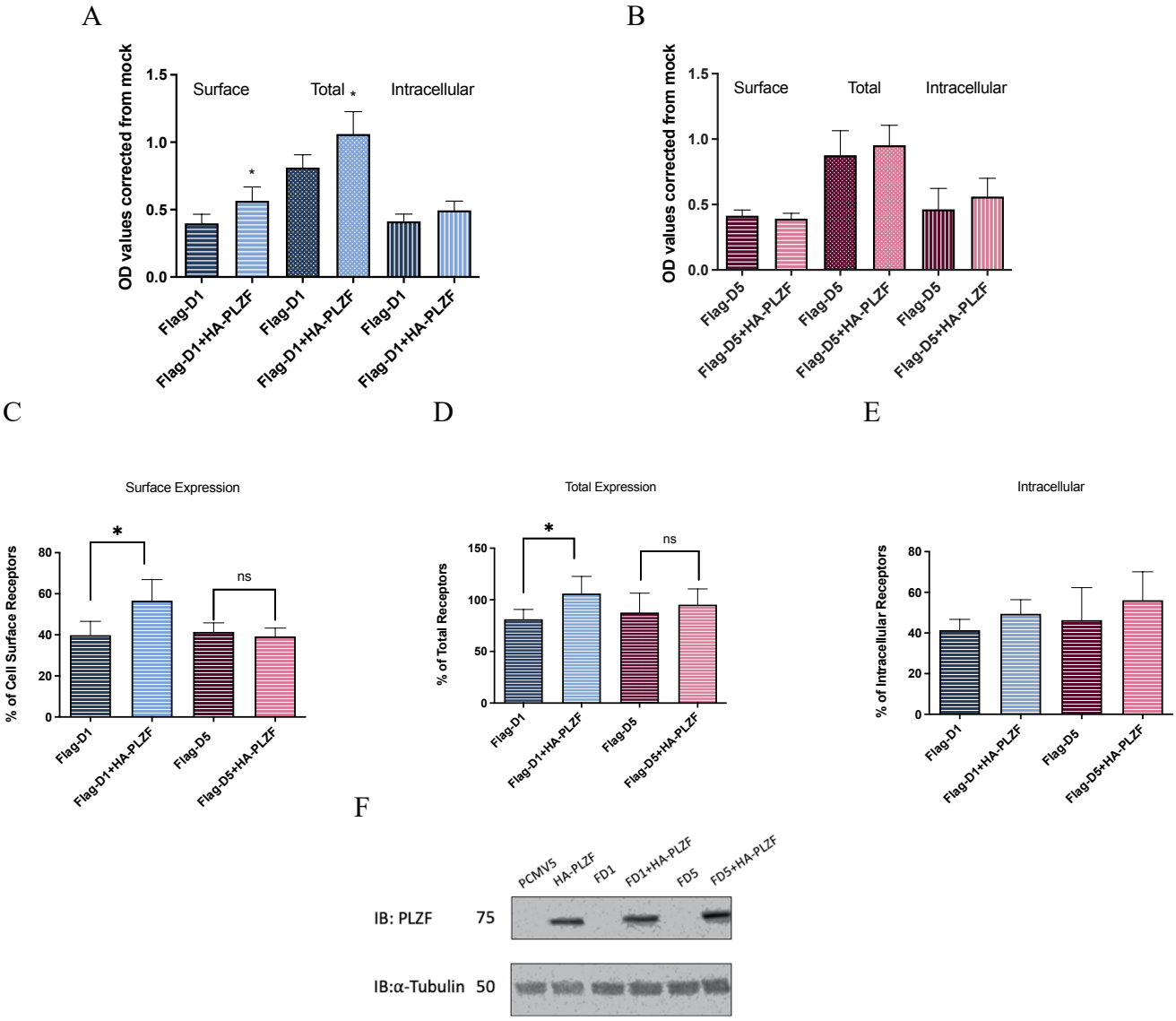


Figure 22. The effect of PLZF on FD1/FD5 total, surface and intracellular expression. ELISA was performed to obtain OD values for HEK293 cells transfected with FD1/FD5±HA-PLZF. OD values were corrected from mock. **A)** FD1±HA-PLZF expression at the total, surface and intracellular levels. N=6 **B)** FD5±HA-PLZF expression at the total, surface and intracellular levels. N=4. **C)** Surface expression of FD1 and FD5 in the presence and absence of HA-PLZF, measured in percentage. **D)** Total expression of FD1 and FD5 in the presence and absence of HA-PLZF, measured in percentage. **E)** Intracellular expression of FD1 and FD5 in the presence and absence of HA-PLZF, measured in percentage. **F)** Representative blots for HEK293 cells transfected with Flag-D1R and Flag-D5R with and without HA-PLZF using anti-PLZF antibodies as described in Materials and Methods. Statistical analysis was performed using column analysis and unpaired t test. B_{max} in pmol/mg proteins for [^{3}H]-SCH23390 expressed as means± SEM for FD1R, FD1R+HA-PLZF, FD5R, FD5R+HA-PLZF were 10.12 ± 0.76, 9.54 ± 0.65, 9.12 ± 0.25, 9.31 ± 0.41.

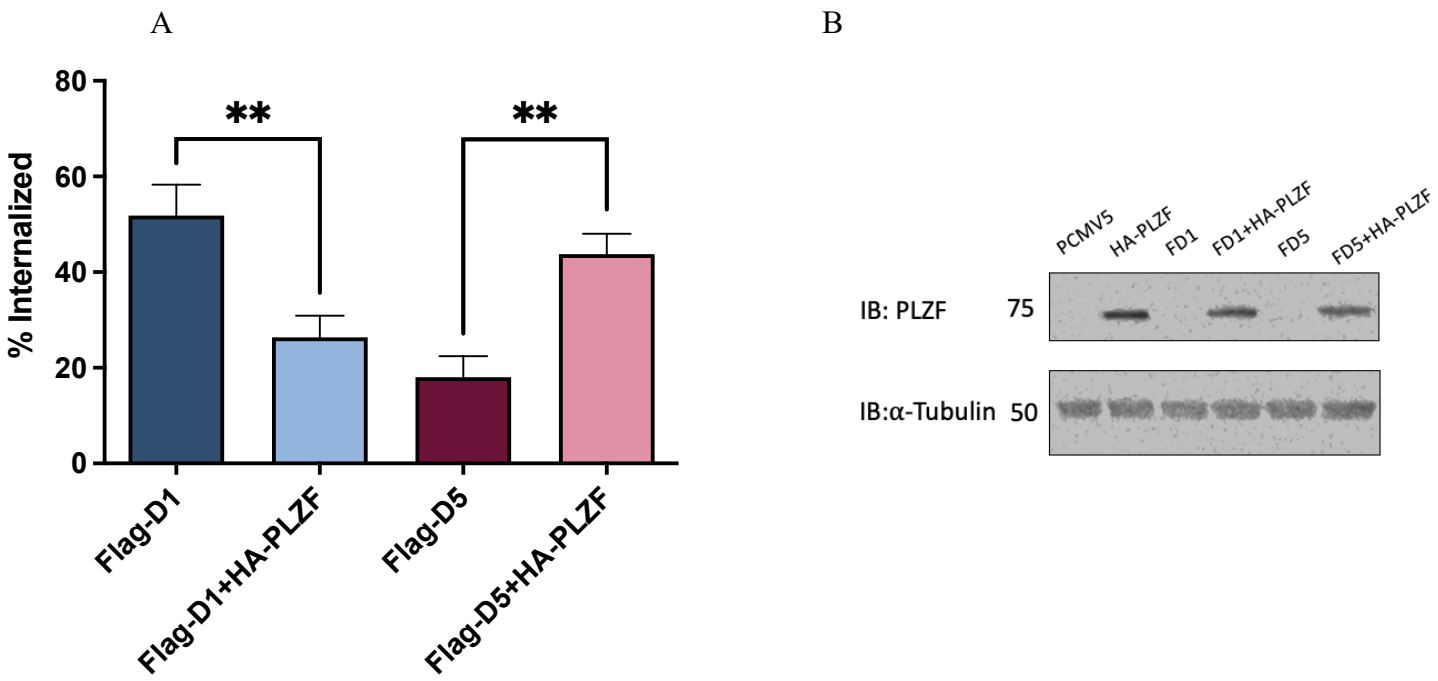
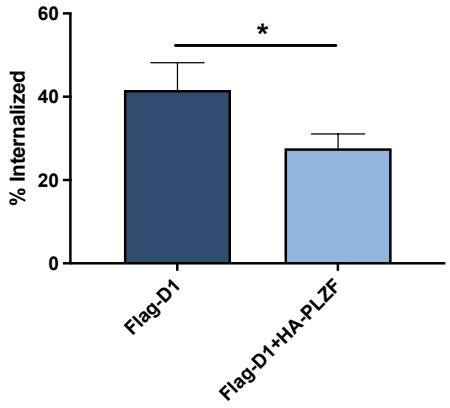


Figure 23. Internalization of FD1/FD5 in the presence and absence of HA-PLZF
ELISA was performed to obtain OD values for HEK293 cells transfected with FD1/FD5±HA-PLZF. OD values were corrected from mock. **(A)** Percentage of FD1±HA-PLZF internalized was calculated. N=9. And percentage of FD5±HA-PLZF internalized was calculated. N=4. Statistical analysis was performed using column analysis and unpaired t-test. **(B)** Representative blots for HEK293 cells transfected with Flag-D1R and Flag-D5R with and without HA-PLZF using anti-PLZF antibodies as described in Materials and Methods. B_{max} in pmol/mg proteins for [^{3}H]-SCH23390 expressed as means ± SEM for FD1R, FD1R+HA-PLZF, FD5R, FD5R+HA-PLZF were 2.04 ± 0.32, 2.14 ± 0.46, 1.21 ± 0.13, 1.05 ± 0.16.

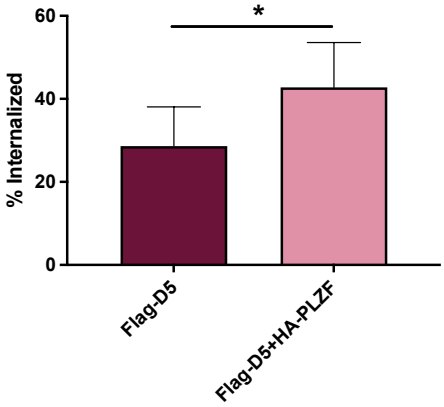
3.3. PLZF oppositely modulates D_1R-class Recycling in HEK293 Cells

To assess whether PLZF modulates the recycling properties of D_1R and D_5R, transfected HEK293 cells were treated with DA for 15 mins, then DA was removed and cells were incubated in an incubation buffer for a series of timepoints (10, 30, 60, and 90 minutes) to allow for the receptor to recycle back to the surface. Results show that PLZF increases the rate of recycling for the D_1R, therefore decreasing the amount of time it takes for the receptor to recycle back to the surface **(Fig. 24C).** At 10 mins, D_1R+PLZF requires a significantly shorter time to recycle back to the cell surface compared to D_1R alone which has not already recycled at this timepoint **(Fig. 24E, F)**. Interestingly, PLZF appears to decrease the rate of recycling for the D_5R and does not allow it to come back to the surface, suggesting that PLZF may divert D_5R trafficking to endosomal degradation **(Fig. 24D).** For D_5R, the receptor eventually recycles at 60 mins and 90 mins, but D_5R+PLZF does not recycle at any of the experimental timepoints **(Fig. 24G, H)**. Thus, PLZF accelerates recovery of the D_1R and decelerates recovery of the D_5R to the cell membrane following the internalization of desensitized receptors that occurs after DA stimulation.

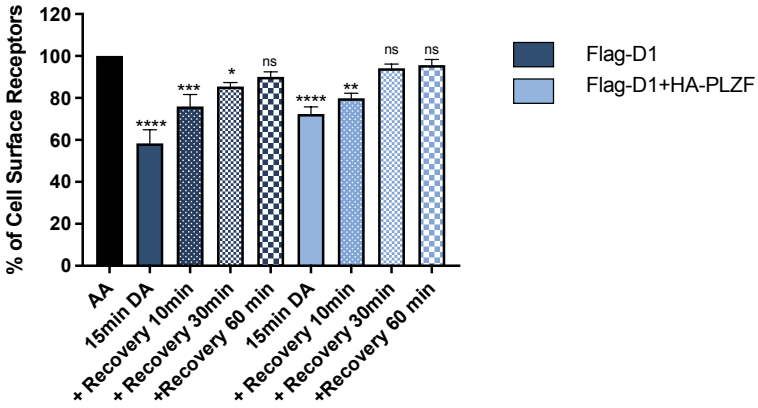
A


% Internalized
60
40
20
0
*
Flag-D1
Flag-D1+HA-PLZF

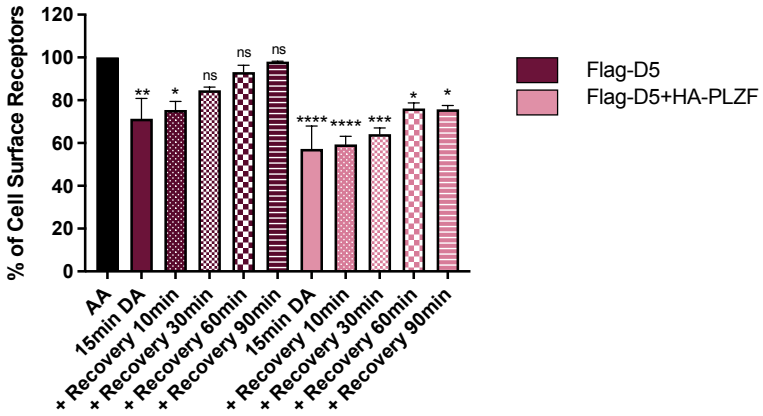
B


% Internalized
60
40
20
0
*
Flag-D5
Flag-D5+HA-PLZF

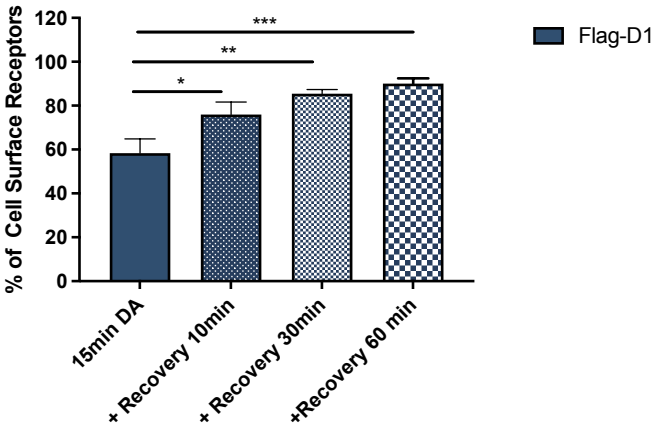
C


% of Cell Surface Receptors
120
100
80
60
40
20
0
****
***
*
ns
****
**
ns
ns
AA
15min DA
+ Recovery 10min
+ Recovery 30min
+Recovery 60 min
15min DA
+ Recovery 10min
+ Recovery 30min
+ Recovery 60 min
Flag-D1
Flag-D1+HA-PLZF

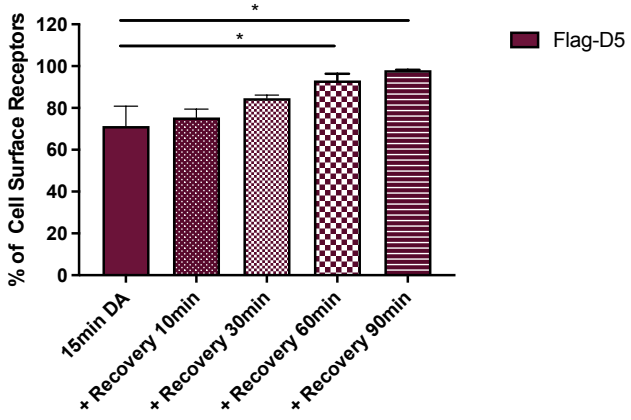
D


% of Cell Surface Receptors
120
100
80
60
40
20
0
**
*
ns
ns
ns
****
****
***
*
*
AA
15min DA
+ Recovery 10min
+ Recovery 30min
+ Recovery 60min
+ Recovery 90min
15min DA
+ Recovery 10min
+ Recovery 30min
+ Recovery 60min
+ Recovery 90min
Flag-D5
Flag-D5+HA-PLZF

E


% of Cell Surface Receptors
120
100
80
60
40
20
0
***
**
*
15min DA
+ Recovery 10min
+ Recovery 30min
+Recovery 60 min
Flag-D1

F


% of Cell Surface Receptors
120
100
80
60
40
20
0
*
*
15min DA
+ Recovery 10min
+ Recovery 30min
+ Recovery 60min
+ Recovery 90min
Flag-D5

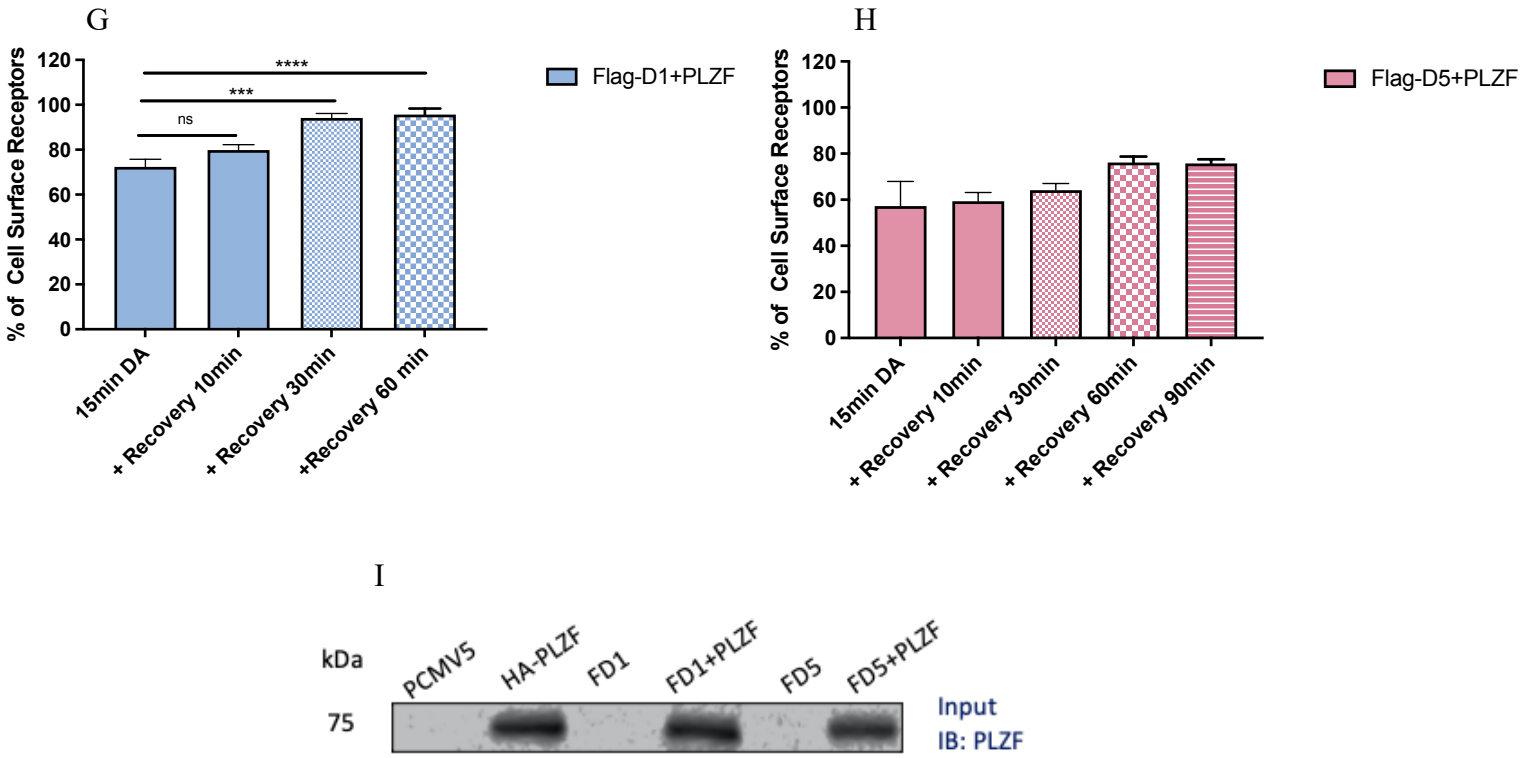


Figure 24. The effect of PLZF on the recycling properties of D1R/D5R.
ELISA was performed to obtain OD values for HEK293 cells transfected with FD1±HA-PLZF N=5 and FD5±HA-PLZF N=3. OD values were corrected from mock. **A and B)** Percent of receptors internalized was calculated for FD1R/FD5R±HA-PLZF and statistical analysis was performed using column analysis and paired t-test. **C and D)** Percentage of cell surface receptors was calculated from the percentage of internalized receptors [100 - % internalized receptors = % cell surface receptors]. **E-H)** Percentage of cell surface receptors for conditions: D1, D1+PLZF, D5, and D5+PLZF, comparing each timepoint to 15 mins DA stimulation. **I)** Representative blots of transfected HEK293 cells, probed with anti-PLZF antibody. Statistical analysis was performed using column analysis and one-way ANOVA. Bmax values in pmol/mg proteins (mean ± SEM) for FD1R, FD1R+HA-PLZF, FD5R, FD5R+HA-PLZF were 2.09 ± 0.72, 1.99± 0.78, 1.50 ± 0.23, 1.63 ± 0.45.

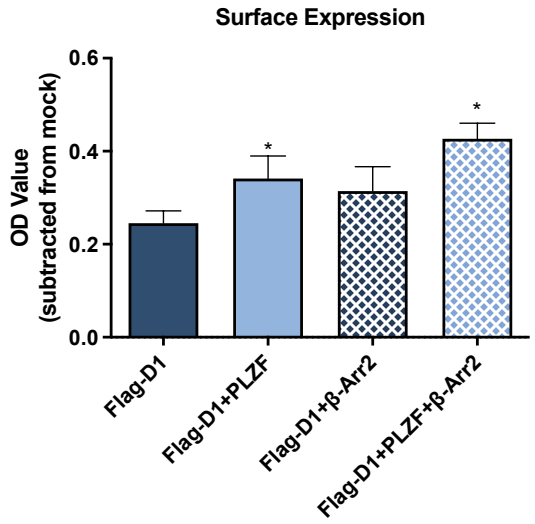
3.4. Overexpression of β-arrestin-2 Disrupts D_5R Internalization and Slightly Facilitates D_1R Surface Expression in HEK293 Cells

Previous results demonstrate the role of PLZF in facilitation of D_5R internalization and in obstructing D_1R internalization. Thus, it is necessary to assess whether PLZF modulates the β-arrestin-dependent internalization of the D_1R-class receptors and the effect of β-arrestin-2 on PLZF mediated D_1R/D_5R internalization. HEK293 cells were transfected with the dominant negative mutant form of β-arrestin (V54D) as well as FD_1R/FD_5R with or without HA-PLZF. The mutant form of β-arrestin competes with the endogenous wild type form and inhibits its actions. The overexpression of β-arrestin-2 (V54D) results in a reduction in the internalization of the D_1R and D_1R+PLZF **(Fig. 25B)**. Similarly, the overexpression of V54D leads to a reduction in D_5R internalization as well as the PLZF-mediated internalization of D_5R **(Fig. 25C)**.

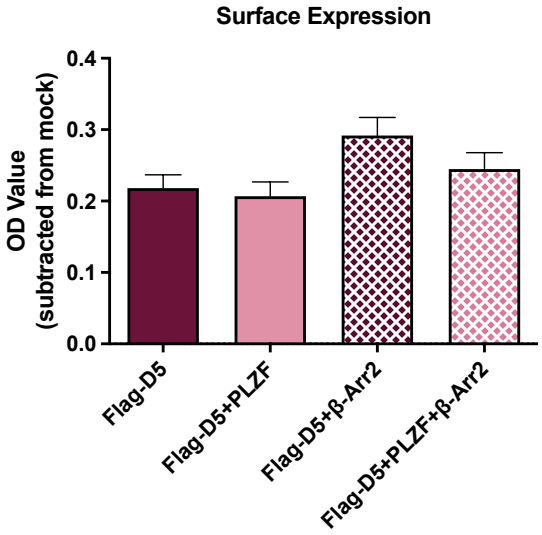
In addition, to determine whether the overexpression of wild type β-arrestin-2 results in any changes in D_1R-class internalization by PLZF, cells were transfected with wild type β-arrestin-2 as well as D_1R/D_5R with or without PLZF. Results show that the overexpression of β-arrestin-2 results in a slight increase in the internalization of the D_1R and D_5R. However, when PLZF is co-transfected with D_1R/D_5R as well as β-arrestin-2, there is a reduction in the internalization of both D_1R and D_5R **(Fig. 25B, C).** PLZF blocks the internalization of D_1R with and without the overexpression of β-arrestin-2, but it appears to hinder the internalization of D_5R when β-arrestin-2 is overexpressed **(Fig. 25B, C).** Interestingly, the overexpression of β-arrestin-2 opposes the facilitated internalization of D_5R by PLZF. In terms of surface expression of D_1R-class, the overexpression of β-arrestin-2 resulted in a slight increase in the surface expression of D_1R, which is also evident when PLZF is present. However, there were no changes in the surface expression of D_5R when β-arrestin-2 was overexpressed **(Fig. 25D-G).**

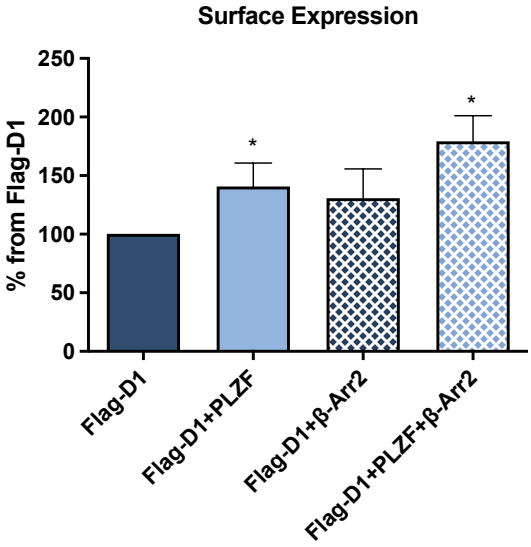
Lastly, co-IP/IB was performed to assess how β-arrestin-2 overexpression effects the interaction between the D_1R-class receptors and PLZF. HEK293 cells were transfected with FD_1R/FD_5R as well as the wild type untagged-β-arrestin-2 in the presence and absence of HA-PLZF. The cells were stimulated using 10 μM DA for 15 minutes. Then the D_1R and D_5R were immunoprecipitated and the associated PLZF and β-arrestin-2 were analyzed by probing with anti-PLZF and anti-β-arrestin antibodies **(Fig. 26).** When PLZF is co-transfected with D_1R/D_5R, β-arrestin-2 signal appears to decrease. Therefore, it is possible that PLZF competes with β-arrestin-2 and results in no significant changes in D_1R-class mediated β-arrestin-2 recruitment under stimulation **(Fig. 27).**

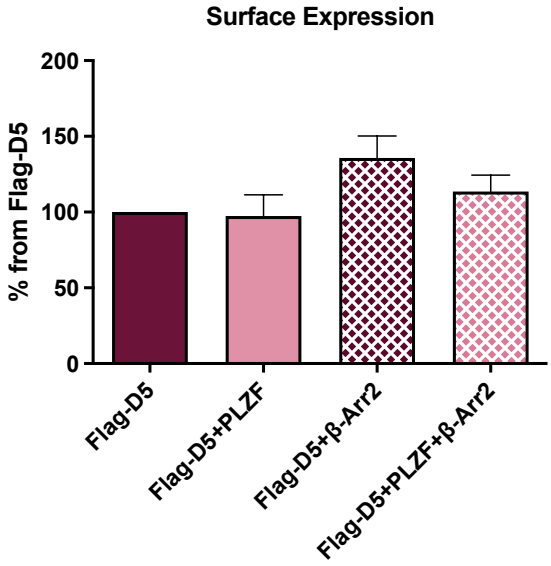
A


Surface Expression
OD Value
(subtracted from mock)
0.6
0.4
0.2
0.0
*
*
Flag-D1
Flag-D1+PLZF
Flag-D1+β-Arr2
Flag-D1+PLZF+β-Arr2

B


Surface Expression
OD Value
(subtracted from mock)
0.4
0.3
0.2
0.1
0.0
Flag-D5
Flag-D5+PLZF
Flag-D5+β-Arr2
Flag-D5+PLZF+β-Arr2


Surface Expression
% from Flag-D1
250
200
150
100
50
0
*
*
Flag-D1
Flag-D1+PLZF
Flag-D1+β-Arr2
Flag-D1+PLZF+β-Arr2


Surface Expression
% from Flag-D5
200
150
100
50
0
Flag-D5
Flag-D5+PLZF
Flag-D5+β-Arr2
Flag-D5+PLZF+β-Arr2

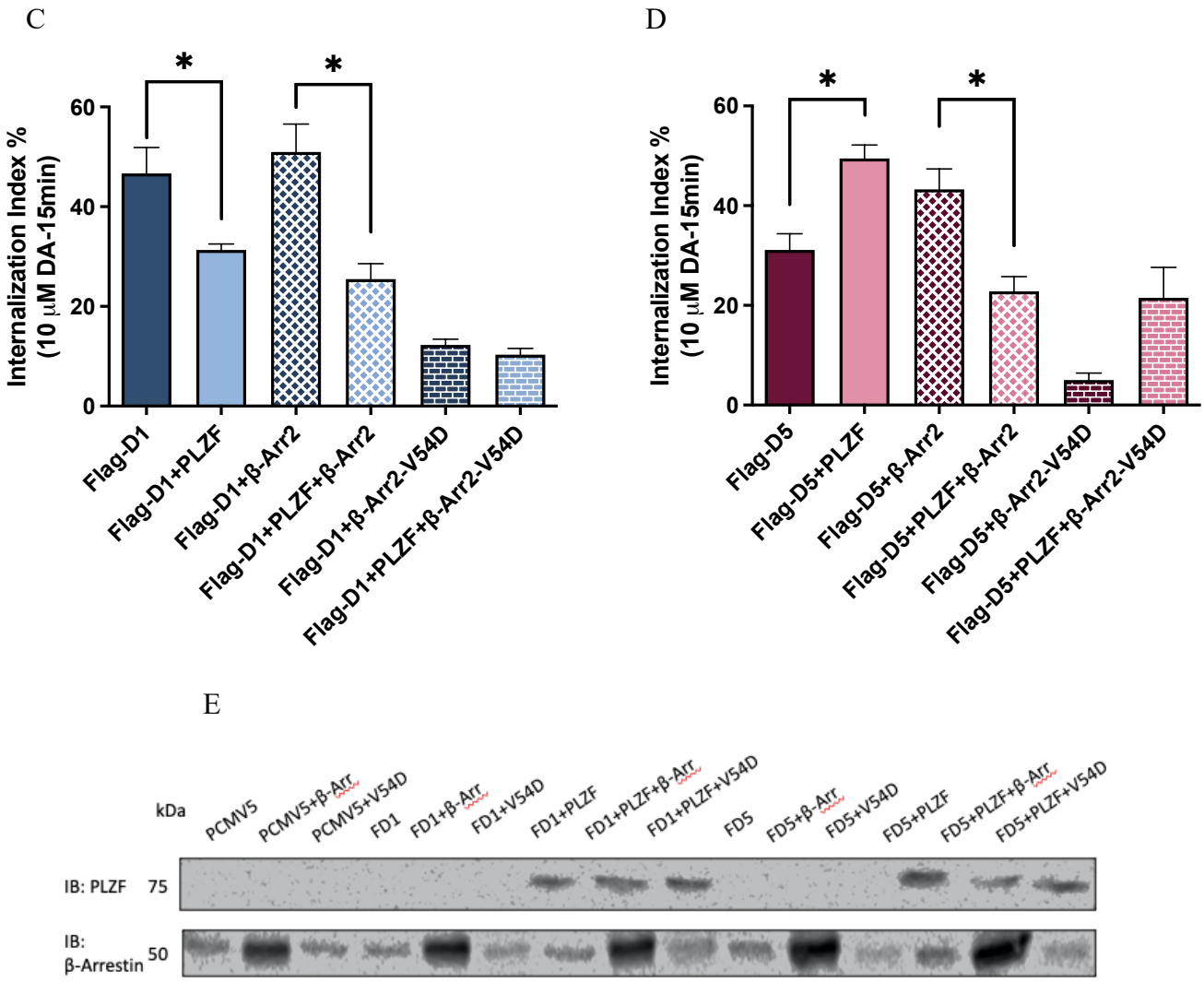


Figure 25. The effect of β-arrestin-2 overexpression on the internalization and surface expression of D1R/D5R.

ELISA was performed to obtain OD values for HEK293 cells transfected with FD1/FD5±HA-PLZF with β-arrestin-2 and V54D. OD values were corrected from mock. **A and B) Top**: OD values for surface expression of D1R and D5R in the presence and absence of PLZF and β-arrestin-2. **Bottom**: Percentage of surface expression for each condition was measured in percent relative to the receptor alone. Statistical analysis was performed using column analysis and one-sample t-test compared to a value of 100. **C and D)** Percentage of internalization was calculated for FD1R±HA-PLZF and FD5R±HA-PLZF with the wild type β-arrestin-2 and mutant form of β-arrestin-2 (V54D). N=4. Statistical analysis was performed using column analysis and one-way ANOVA (*$p < 0.05$). **E)** Representative blots of transfected HEK293 cells, probed with anti-PLZF and anti-β-arrestin-2 antibodies. Bmax values in pmol/mg proteins (mean ± SEM) for FD1R, FD1R+HA-PLZF, FD1R+β-arr, FD1R+HA-PLZF+β-arr, FD5R+β-arr, FD5R+HA-PLZF+β-arr, FD1R+V54D, FD1R+HA-PLZF+V54D, FD5R+V54D, FD5R+HA-PLZF+V54D were 2.91 ± 0.82, 2.35± 0.64, 1.76 ± 0.54, 1.81 ± 0.76, 2.05 ± 0.55, 2.76 ± 0.78, 2.33 ± 0.43, 3.09 ± 0.87, 2.86 ± 0.45, 2.77 ± 0.54.

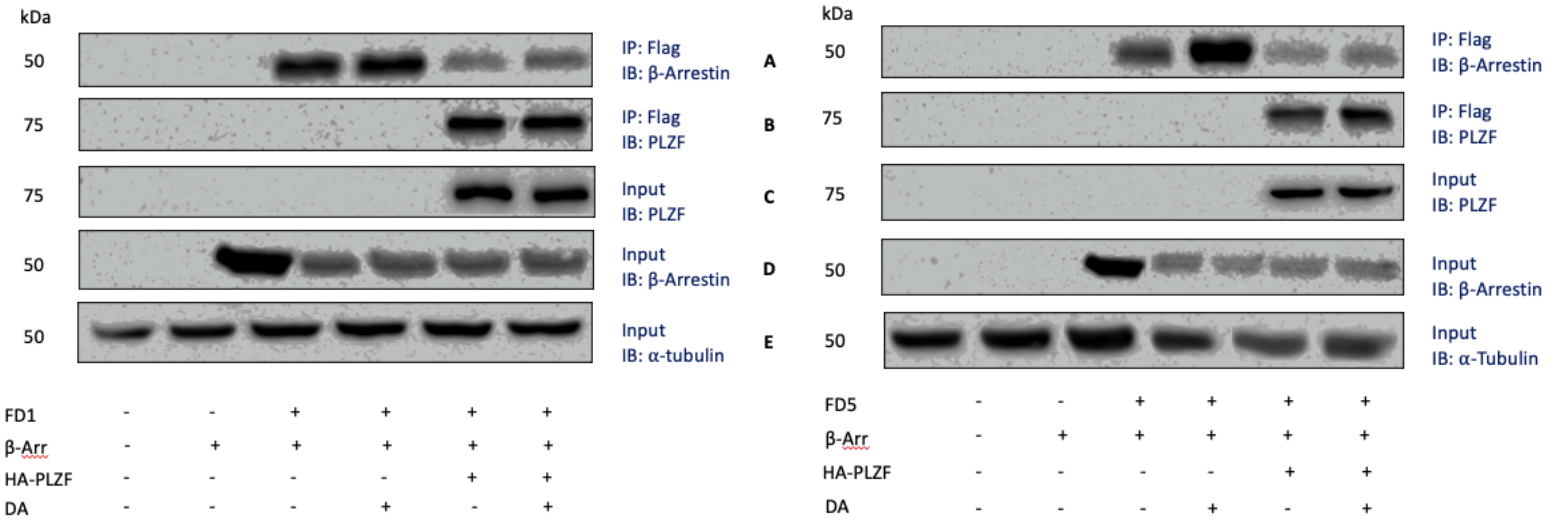


Figure 26. Complex formation between FD1/FD5 ± HA-PLZF and β-Arrestin-2 under basal and DA stimulation.

Representative experiment of co-immunoprecipitation and immunoblotting of HEK293 cells transfected with FD1 or FD5 with β-Arrestin-2 and HA-PLZF then subject to 15 mins stimulation with vehicle or DA. FD1 and FD5 were immunoprecipitated using anti-Flag conjugated beads and probed using **A)** anti-β-Arrestin antibody as well as **B)** anti-PLZF antibody. Inputs were probed using **C)** anti-PLZF antibody, **D)** anti-β-Arrestin antibody and **E)** anti-alpha tubulin antibody. N=4.

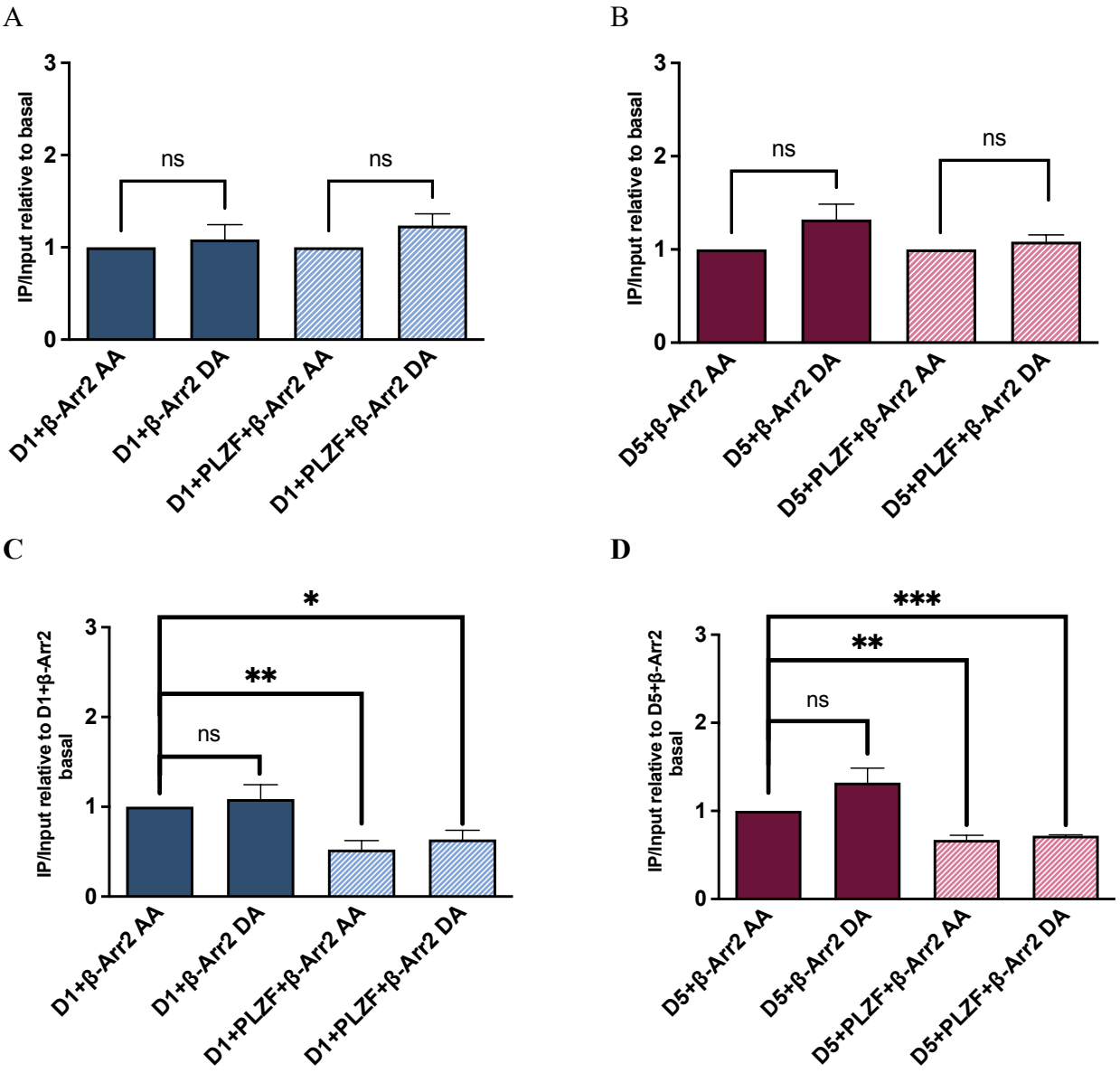


Figure 27. Quantification of the effect of DA stimulation on complex formation between FD1/FD5 ± HA-PLZF and β-Arrestin-2.

Densitometric analysis using GelQuant software for the co-immunoprecipitation of **A+C)** FD1± HA-PLZF with β-Arrestin-2 and **B+D)** FD5± HA-PLZF with β-Arrestin-2 under stimulation of HEK293 cells using 10 µM DA for 15 mins, relative to basal of D1+β-Arr2 and D1+PLZF+β-Arr2 **(A+B)**, and relative to D1+β-Arr2 basal **(C+D)**. Statistical analysis was performed using column analysis and one sample t-test compared to a value of 1. Bars represent mean ± SEM. N=4. Bmax values in pmol/mg proteins (mean ± SEM) for FD1, FD1+HA-PLZF, FD5, and FD5+HA-PLZF were 6.23 ± 0.98, 5.14 ± 0.92, 5.01 ± 0.83, and 4.62 ± 0.97.

DISCUSSION

Many efforts have been devoted to investigate the full complement of signaling pathways mediated by individual DAR subtypes. The D_1R-class are of considerable interest because they are the most abundant DARs in the CNS (Goldman-Rakic, Muly, & Williams, 2000). Recent work suggests that DA neurotransmission could be regulated via DRIPs, which include cytoskeletal, adaptor and signaling proteins (Bergson et al., 2003). Therefore, it is of pivotal importance to gain further understanding of the molecular mechanisms underlying DA-related CNS disorders by investigating the role of DRIPs in regulation of the D_1R-class. Studies have demonstrated that various proteins may play a role in receptor trafficking, membrane insertion and anchoring, as well as the aggregation and immobilization of receptors (Wang & Liu, 2008).

A valid method for identifying such protein interactions is the yeast two-hybrid screen, which involves screening the protein products of a cDNA library with a selected domain derived from a GPCR (Tanowitz & von Zastrow, 2004). This method has served to extend our view on the classical paradigm of GPCR signaling as it has revealed that there are non-G protein DAR associated proteins which are crucial in the regulation of these receptors (Tanowitz & von Zastrow, 2004). Thus, our lab used this proteomic approach to identify potential binding partners for the highly homologous D_1R and D_5R using the IL3 and CT regions as baits in yeast two-hybrid screens of a human adult brain cDNA library. We identified PLZF as a binding partner that interacts in a subtype-specific fashion with the IL3 of D_5R and with CT of D_1R. Following these findings, our lab wanted to validate whether this was worth pursuing further and my MSc project served to establish the significance of the PLZF protein interacting with DARs from the same class but with different cytoplasmic domains.

Therefore, to demonstrate the role of PLZF as a novel regulator of the D_1R and D_5R, I set out to uncover its role in receptor trafficking and signaling mechanisms. My project results

demonstrate that PLZF associates with the D_1R-class in HEK293 cells that were co-transfected with PLZF and either D_1R or D_5R. Stimulation of the D_1R-class receptors results in an enhanced association of the receptor with PLZF in vitro. In addition, the co-expression of PLZF and β-arrestin 2 with the D_1R or D_5R suggests that PLZF may reduce β-arrestin 2 recruitment to the receptors. Functional assays indicate that PLZF increases D_1R-stimulated cAMP production but decreases D_5R-stimulated cAMP production. Similarly, PLZF increases D_1R-mediated ERK activation and decreases D_5R-mediated ERK activation. Interestingly, PLZF increases surface and total expression, yet attenuates agonist-mediated D_1R internalization. In contrast, PLZF has no significant effect on D_5R surface and total expression but facilitates receptor internalization. Lastly, PLZF accelerates recovery of the D_1R to the cell membrane following internalization of desensitized receptors but delays recovery of the D_5R.

1. Agonist-modulated conformation of the D_1R and D_5R could render the receptor more accessible for PLZF binding

Primarily, the radioligand binding saturation and competition studies suggest that the interaction between PLZF and the cytoplasmic domains of the D_1R and D_5R does not impact the ability for the agonists, antagonist or inverse agonist to modulate the affinity of the receptors **(Fig. 18, 19)**. Thus, in cells overexpressing the D_1R or D_5R, it appears that the constitutive interaction does not alter the ligand binding pocket of each receptor. Based on this observation and after establishing the specific positive interaction with the yeast two-hybrid system, it is important to demonstrate further that this interaction is likely to occur in vitro using full length receptors and co-IP studies.

The interaction between the D_1R or D_5R and PLZF was assessed with respect to the recruitment of PLZF to the complex. Although there is no change in the ligand binding affinity of these receptors, the different treatments of *cis*-flupentixol (inverse agonist) and DA (natural agonist) resulted in alterations in PLZF recruitment **(Fig. 17)**. It is possible that the *cis*-flupentixol stimulation promotes an inactive conformation of the receptors as there is an observed reduction in PLZF recruitment while DA stimulation results in the stabilization of the receptor in an active state and further recruitment of PLZF to the complex. Rather than the early view of GPCRs as binary on-off switches, our current understanding of receptors as dynamic proteins which are able to sample multiple conformational states serves to explain the interaction between D_1R-class and PLZF (Mahoney & Sunahara, 2016). When the *cis*-flupentixol or DA binds to the receptors, there is a conformational plasticity which allows the receptor to interact with numerous signaling partners to produce spatially and temporally textured signals (Mahoney & Sunahara, 2016).

To better characterize the association between the D_1R-class and PLZF, it is important to understand the ternary complex model which explains the link between the receptor, agonist and the G protein (De Lean, Stadel, & Lefkowitz, 1980). This model suggests that GPCRs are in a state of equilibrium between two inter-convertible states, an inactive state (R) which binds agonists with low affinity and an active state (R*) which binds agonists with high affinity where G protein solely binds to the R*. The agonists promote changes in the inactive receptor conformation and these changes allow G protein binding and receptor activation by stabilizing the receptor in the R* state, which results in a maximal response. However, the inverse agonists shift the equilibrium toward the R state and maintain the receptor in the inactive conformation.

On the contrary, the antagonists act by blocking the binding of the receptor to other ligands and have an equal affinity for both the R and R* states (Park et al., 2008).

Therefore, based on this pharmacological model, an agonist such as DA would activate the D_1R and D_5R, stabilizing the receptors in the R* state, and the inverse agonist such as *cis*-flupentixol would maintain the receptors in an inactive (R) conformation. The co-IP studies demonstrate an increase in PLZF recruitment upon DA stimulation of the receptors which suggests that PLZF has increased avidity for the D_1R-class when the receptors are in the active conformation, due to DA stimulation **(Fig. 17)**. As a result, it can be assumed that PLZF is always associated with the D_1R and D_5R which may be explained by the presence of a pool of receptors and this pool of either the D_1R or D_5R are maintained in a state which does not allow PLZF to bind to them. The DA treatment may then allow this pool of either the D_1R or D_5R to change conformation and consequently become competent for PLZF interaction. Therefore, under the basal state, it is possible that the PLZF dissociates and associates as the receptor is always dynamically changing conformation and flipping back and forth from inactive to active form. When the receptor is treated with a drug, such as *cis*-flupentixol or DA, there is a stabilization of the receptor in one conformation over another, and PLZF seems to favour the active state of both the D_1R and D_5R.

Moreover, GRKs specifically recognize the active or agonist-occupied form of the receptor and terminate G protein-dependent signaling by phosphorylating the active GPCRs at their CT and/or intracellular loops, allowing arrestin binding (Carman & Benovic, 1998; Kaya et al., 2020). β-arrestin binds to the phosphorylated and activated receptors with high affinity, which simultaneously inhibits further G protein coupling or desensitization, and triggers receptor internalization. The nature of cellular signaling is complex and is not divided into two distinct branches: G protein dependent or β-arrestin dependent. Alternatively, it is more reasonable that

receptor-β-arrestin and receptor-G protein complexes coexist in a population in the cell (Gurevich & Gurevich, 2006; Peterson & Luttrell, 2017). This coexistence allows different phosphorylation patterns, which may result in different affinities for β-arrestin.

Interestingly, studies showing the crystal structures of the receptor-β-arrestin complexes reveal that β-arrestin binds to both the phosphorylated elements in the CT and the main G protein binding site (Huang et al., 2020; Staus et al., 2020; Yin et al., 2019; Zhou et al., 2017). In relation to the D_1R-class, studies have shown that the different phosphorylation patterns of the D_1R affect the $G\alpha_s$ coupling specifically in the presence of β-arrestin 2 (Kaya et al., 2020). Therefore, this effect of phosphorylation pattern or "barcode" on β-arrestin 2 coupling may indirectly effect the $G\alpha_s$ coupling (Kaya et al., 2020) **(Fig. 28)**. This model is relevant to my findings as our lab has identified PLZF as a binding partner for the D_1R at the CT and for D_5R at the IL3. Therefore, the differences in the observed results could be due to the location of the interaction and the phosphorylation status of each receptor upon agonist and PLZF binding to each receptor.

For instance, it was previously reported that the β_1-adrenergic receptor (β_1AR) is associated with PSD-95 through a PDZ domain-mediated interaction, and is regulated by GRK5. The GRK5 regulates β_1AR association with PSD-95 through phosphorylation of β_1AR (Hu et al., 2002). Therefore, the regulation of protein association through receptor phosphorylation may be a general mechanism used by GPCRs that associate via PDZ domain-mediated protein-protein interactions (Sheng & Sala, 2003). Thus, D_1R-class phosphorylation could dictate the differential regulation of PLZF for each receptor subtype. Although the D_1R and D_5R are structurally related and contain a similar number of Ser and Thr phosphorylation sites on their cytoplasmic regions, PLZF interacts with a different region in each receptor. It is likely that the PLZF interactions

with the CT of the D_1R and the IL3 of the D_5R are regulated by GRKs and the GRK-mediated phosphorylation results in a change in conformation of each receptor (Pitcher, Freedman, & Lefkowitz, 2003). This change of conformation may allow an increase in PLZF interaction when an agonist is bound and leads to the observed results for each receptor subtype.

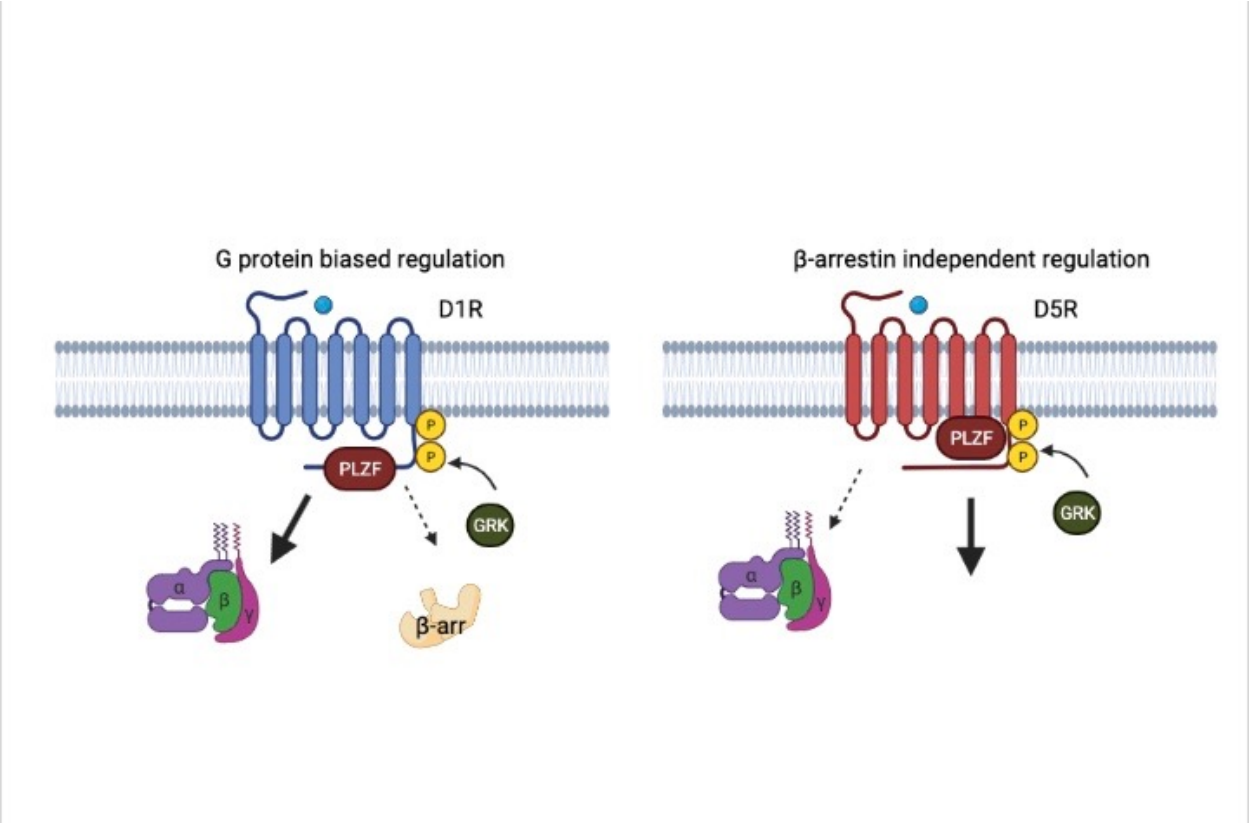


Figure 28. Schematic diagram of the G protein biased regulation and β-arrestin independent regulation of D_1R-class interaction with PLZF
PLZF interaction with the CT of the D_1R and the IL3 of the D_5R may promote an active conformation of the receptor that favours either G protein biased or β-arrestin independent regulation targeting for endocytosis. This may result in the observed contrasting modulation of the cAMP/ERK activation and internalization.

2. PLZF differentially regulates D_1R-class endocytosis

The co-IP experiments performed on HEK293 cells co-expressing PLZF and β-arrestin-2 reveals that there is no interaction between the two proteins (data not shown due to blank WB gel), which may be due to both proteins lacking the binding sites or common interactor that enable a direct interaction between the PLZF and β-arrestin-2. Although β-arrestin-2 increases the internalization of both the D_1R and D_5R, the co-IP studies reveal that in comparison to the co-expression of the D_1R or D_5R with β-arrestin-2, the transfection of D_1R or D_5R with β-arrestin-2 and PLZF results in a reduction of β-arrestin-2 interaction with the D_1R and D_5R under basal state as well as DA stimulation **(Fig. 27)**. Therefore, it is important to note that anytime the D_1R or D_5R is co-expressed with PLZF, there is a decrease in β-arrestin-2 recruitment to the receptors. The results from this experiment suggest that perhaps the role of PLZF is to compete with β-arrestin-2 and if PLZF can compete, the receptor resides at the cell surface for a longer time.

Furthermore, at 15 minutes, the D_1R or D_5R alone may attain the extent of internalization observed for β-arrestin-2 and D_1R/D_5R **(Fig. 25)**. However, since this observation only covers the 15 mins timeframe, it is possible that the β-arrestin-2 affects the rate of internalization for each receptor which needs to be validated in future studies. When PLZF is co-expressed with β-arrestin-2 and the D_1R or D_5R, there is a decrease in the extent of internalization for each receptor in comparison to the cells expressing β-arrestin-2 and D_1R or D_5R. Therefore, in relation to the co-IP results, PLZF opposes β-arrestin-2 effect on internalization and impedes the extent of internalization for each receptor. Analysis of the effect of PLZF on the dominant negative mutant form of β-arrestin-2, known as β-arrestin-2-V54D, demonstrates that the mutant form

V54D impedes D_1R and D_5R endocytosis and PLZF does not seem to alter this impediment or rescue the V54D mutant effect on endocytosis **(Fig. 25)**.

In addition, when the D_1R or D_5R is co-expressed with PLZF, PLZF appears to impede D_1R internalization while promoting D_5R internalization **(Fig. 23)**. This contrasting difference in the endocytosis of the receptor may be possibly due to the location of the interaction for each receptor. As discussed earlier, PLZF may interfere with the GRK-mediated phosphorylation of the receptor resulting in changes in β-arrestin-2 affinity to the receptor. Since PLZF seems to block D_1R endocytosis, it is possible that this D_1R-PLZF interaction at the plasma membrane results in lower affinity pattern for β-arrestin-2 binding. Therefore, internalization is inhibited and receptor-G protein complexes are favoured. However, PLZF behaves differently with the D_5R as internalization is facilitated, resulting in β-arrestin-2-independent regulation.

3. PLZF may modulate D_1R- and D_5R-mediated cAMP and ERK1/2 levels as a result of IL3 and CT dependent interactions

Primarily, it is important to note that transfection of PLZF alone had no effect on cAMP levels. The observed enhancement of D_1R-mediated cAMP and ERK1/2 accumulation by PLZF co-expression could be mediated by an increase in the residence time of the D_1R at the cell surface as well as the impaired internalization of the D_1R **(Fig. 20, 21)**. In contrast, PLZF decreases the D_5R-mediated cAMP and ERK1/2 activation possibly due to the receptor being rapidly removed from the cell surface **(Fig. 20, 21)**. These detected changes in D_1R- and D_5R-mediated cAMP production may also be due to PLZF regulation of G protein coupling to the receptor leading to cAMP production and/or uncoupling from activated receptor resulting in desensitization. Although the B_{max} for the D_1R was consistently unchanged by PLZF, there is a decrease in the B_{max} of D_1R+PLZF compared to D_1R **(Fig. 21).** Since PLZF increases D_1R-

mediated ERK1/2 activation, the decrease in the B_{max} of D_1R+PLZF compared to D_1R further validates that this observed increase in ERK1/2 activation is due to the effect of PLZF on the D_1R-mediated ERK1/2 activation rather than the total density or concentration of the D_1R.

Interestingly, *eor-1* was previously identified as the C. elegans ortholog of mammalian PLZF and *eor-1* has been shown to positively regulate Ras signaling during excretory system development (Howard & Sundaram, 2002; Rocheleau et al., 2002). The *eor-1* has been shown to function downstream or in parallel to ERK and encodes nuclear proteins that likely act at the level of transcriptional regulation (Howard & Sundaram, 2002; Rocheleau et al., 2002). Specifically, it has been shown that the *eor-1* could be a downstream target of the Ras/ERK pathway or could cooperate with the Ras/ERK pathway to promote certain cellular outcomes (Howard & Sundaram, 2002; Rocheleau et al., 2002). Therefore, this characteristic positive regulation of the Ras/ERK signaling could be relevant to the PLZF interaction with the D_1R which caused an increase in ERK activation.

As shown previously, PLZF impedes D_1R internalization and the D_1R occupies the cell surface for a longer period of time, which may strengthen the coupling with the $G\alpha_s$. In addition, GRK-mediated phosphorylation may also be dictating the manner by which PLZF interacts with the receptor. For instance, the D_1R with PLZF may require more time to be desensitized by virtue of remaining in the plasma membrane for longer time. Since PLZF interacts with the CT of the D_1R and the CT is known to be the site of phosphorylation, PLZF may preclude GRK to phosphorylate the D_1R and recruit β-arrestin-2. This interpretation is also in line with the co-IP experiments demonstrating reduced β-arrestin-2 recruitment when PLZF is co-expressed with the D_1R. In contrast, the interaction between PLZF and the IL3 of D_5R facilitates internalization,

which may result in a disruption in $G\alpha_s$ coupling causing a decrease in cAMP and the downstream ERK activation **(Fig. 29)**.

In addition, studies revealed that PLZF is a G protein alpha subunit o ($G\alpha_o$)-binding partner and it directly interacts with the $G\alpha_o$ subunit to regulate its activity (Won et al., 2008). Specifically, the $G\alpha_o$ GTPase domain, which facilitates the sequential dissociation of Gα from the Gβγ dimer and allows the release of these G protein subunits to interact with various downstream effectors, is important in modulating the function of PLZF (Won & Ghil, 2009). The $G\alpha_o$ is known as one of the most abundant proteins in the CNS and pathogenic mutations in its gene (*GNAO1*) causes movement disorders (Muntean et al., 2021). Recently, a study has found that the $G\alpha_o$ subunit does not directly regulate cAMP-producing activity of AC5 in striatal membranes but rather regulates cAMP production by managing the availability of Gβγ for AC5 binding (Muntean et al., 2021). With this mechanism in mind, the PLZF interaction with the $G\alpha_o$ subunit could be relevant to the D_1R-class interaction with PLZF. It is possible that the PLZF interaction with the D_1R-class results in alterations to G-protein coupling or could be a result of an increase in Gβγ availability and therefore a subsequent increase in AC and cAMP.

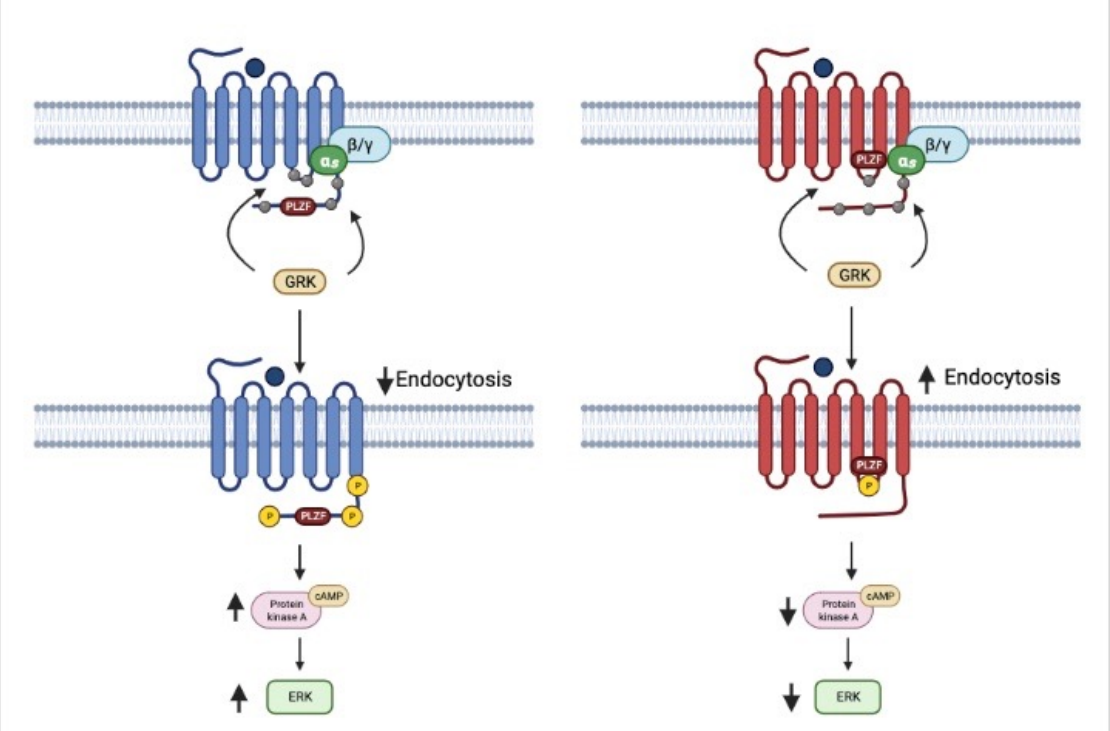


Figure 29. Working model of GRK-mediated phosphorylation and barcoding of the D_1R-class interaction with PLZF

The D_1R and D_5R each possess unique phosphorylation sites that are targets for GRK phosphorylation. PLZF binds to the CT of the D_1R and the IL3 of the D_5R to possibly regulate the GRK-mediated phosphorylation of each receptor. The altered phosphorylation code is sensed by the recruited β-arrestin resulting specific conformational orientations of β-arrestin and alterations in endocytosis and G protein activation. These alterations lead to increased D_1R- and decreased D_5R-mediated cAMP and ERK activation.

4. PLZF distinctly regulates post-endocytic trafficking of D_1R and D_5R

Although there may be different arrestin affinity patterns that exist with the presence of PLZF and the co-IP experiment reveals less β-arrestin-2 recruitment to the complex, the ELISA recycling data reveals that there may also be a distinct post-endocytic pathway for the D_1R and the D_5R. GPCRs undergo significant endocytosis from the plasma membrane in response to agonist stimulation. It is known that receptor endocytosis of desensitized GPCRs is an initial step in the resensitization process that requires ligand-receptor dissociation and receptor dephosphorylation. In some cases, receptors are recycled back to the plasma membrane, but other times the receptors are targeted to lysosomes for degradation (Hanyaloglu & Von Zastrow, 2008). GPCR internalization is greatly influenced by two of the canonical families of GPCR-interacting proteins, the GRKs and arrestins (Reiter & Lefkowitz, 2006). The recovery at the membrane of internalized receptors has been linked to the synthesis or insertion of more receptors, or to the recycling of endocytosed receptors (Wang et al., 2017). There are a few DRIPs which have been shown to regulate this process including PSD-95, β-arrestin, GRK, and GSK-3β (Lee et al., 2002; Lefkowitz & Shenoy, 2005; Maurice et al., 2011; Sun et al., 2009; Von Zastrow & Williams, 2012; Walther & Ferguson, 2013; Wang et al., 2017). Previous studies have shown that the cytoplasmic protein DRiP78 interacts with a sequence in the proximal CT of the D_1R to promote export of newly synthesized receptors from the ER (Bermak et al., 2001). Furthermore, a study has identified a sequence spanning amino acid residues 360-382 present in the CT cytoplasmic domain of the human D_1R that is not required for agonist-induced endocytosis of receptors but is required for efficient recycling of internalized receptors to the plasma membrane (Vargas & Von Zastrow, 2004). It was shown that this D_1R-derived sequence is sufficient to induce rapid recycling and prevent lysosomal proteolysis when fused to the distal

CT of the δ opioid receptor (Tsao & Von Zastrow, 2000; Vargas & Von Zastrow, 2004). Thus, the PLZF protein may interact with the sequence bounded by residues 360-382 of the D_1R to mediate recycling of the receptor from the endocytic pathway. In addition, the co-IP studies reveal that PLZF may compete with the β-arrestin-2 in the endocytosis of the D_1R leading to decreased internalization of the receptor yet faster recycling rate of the D_1R **(Fig. 24, 30)**.

In contrast, PLZF may play a different role in the post-endocytic trafficking of the D_5R as it appears to delay the recovery or recycling of the receptor to cell membrane **(Fig. 24)**. It is important to note that it has not been investigated whether the overexpression of β-arrestin-2 would accelerate the recovery of the D_5R as this would indicate that there are two recycling pathways and PLZF favours one over the other. However, based on the obtained data, I hypothesize that the D_5R exists as two pools of receptors that are either bound to PLZF or bound to β-arrestin-2. Therefore, it is possible that as PLZF is overexpressed, the β-arrestin-2 is no longer the dominant endocytic mediator and PLZF accompanies the D_5R through a distinct post-endocytic pathway rather than the β-arrestin-2 pathway. Since PLZF contains a ENTH binding domain and epsin has been shown to bind to clathrin through direct association with the clathrin-terminal domain, it is possible that PLZF also plays a major role in the clathrin-mediated endocytosis of the D_5R (Drake et al., 2000).

Essentially the internalized cargo converges into a common early endosome, which are then sorted for recycling or degradation. Recycling of receptors back to the plasma membrane can occur directly from the early endosome (the fast recycling pathway) or indirectly through a distinct subpopulation of recycling endosomes (the slow recycling pathway) (O'sullivan & Lindsay, 2020). Therefore, the cargo that is internalized from the plasma membrane is sorted in the early endosome and can subsequently either progress along the degradative pathway or return

to the plasma membrane (O'sullivan & Lindsay, 2020). At this point, the cell may need to make a sorting decision in which a late endosome eventually becomes a recycling endosome or proceeds to the lysosome for degradation. PLZF may favour the late endosomal pathway which results in the observed delayed recycling of the D_5R or could eventually lead to the degradation of the D_5R **(Fig. 31)**. This mechanism remains to be investigated as the obtained data does not reveal whether the source of the recovered D_5R is from the recycling endosomes or if these recovered D_5R are de novo synthesis receptors.

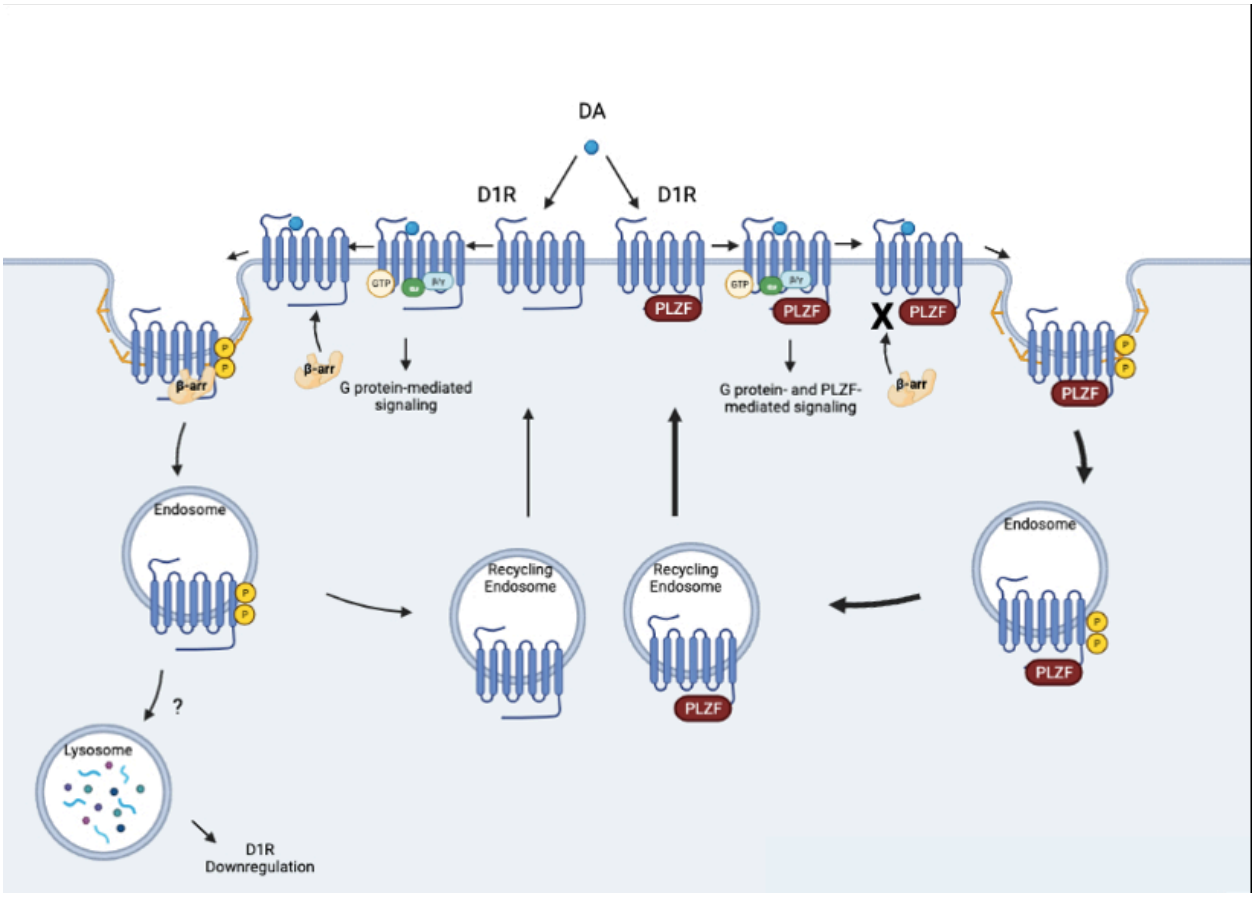


Figure 30. The interacting protein PLZF may regulate the post-endocytic trafficking of the D_1R

Following agonist-induced receptor endocytosis, some DARs are targeted for lysosomal degradation, while others are rapidly recycled back to the plasma membrane. The interaction between PLZF and the D_1R promotes a faster recycling pathway of the receptor. The lysosomal pathway may be possible under long term stimulation (hours).

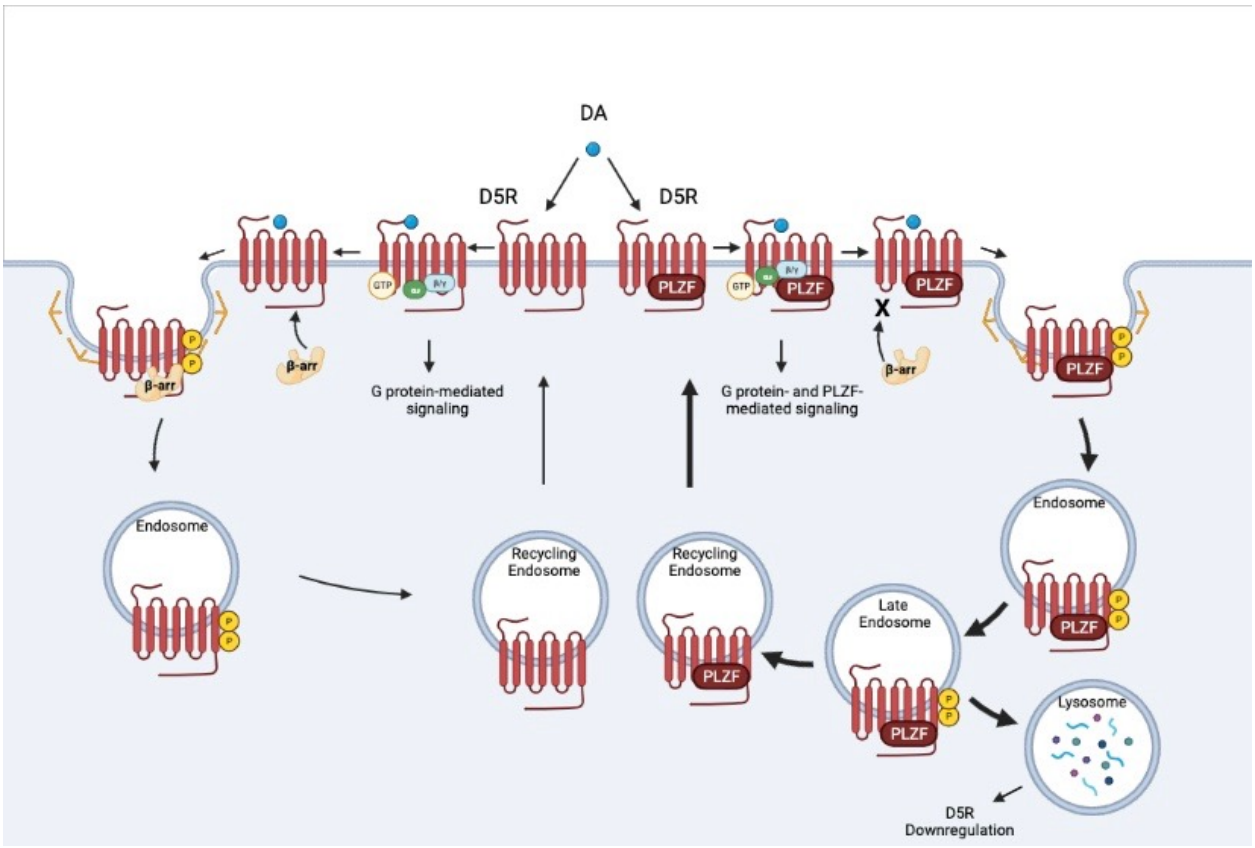


Figure 31. The interacting protein PLZF may regulate the post-endocytic trafficking of the D_5R

Following agonist-induced receptor endocytosis, some DARs are targeted for lysosomal degradation, while others are rapidly recycled back to the plasma membrane. The interaction between PLZF and the D_5R promotes delayed recycling of the receptor (thick arrows). The delayed recycling of the receptor is due to the presence of the late endosome. The cell then undergoes a sorting decision where the receptor is either recycled back to the plasma membrane or downregulated through the lysosomal degradation.

5. Future Directions

Interestingly, a study has revealed that GSK-3β is a novel interacting protein for the D_1R at the CT and attenuates D_1R internalization, resulting in D_1R dysfunction (Wang et al., 2017). This enzyme is known to have broad actions in the regulation of cellular function and its activity is regulated by serine (inhibitory) and tyrosine (stimulatory) phosphorylation (Wang et al., 2017). Similarly, PLZF plays a role in cellular function and its activity could be regulated by tyrosine phosphorylation. As revealed in the study demonstrating the PLZF association with the AT2R, AngII stimulation induced tyrosine phosphorylation of PLZF allowing nuclear translocation of PLZF (Senbonmatsu et al., 2003). Therefore, it is possible that the phosphorylation of PLZF may play a role in the agonist-stimulated D_1R or D_5R endocytosis. In other words, the differential regulation that is imparted on the D_1R and D_5R could also be at the level of the activity of PLZF. Therefore, detecting the phosphorylation of the D_1R-class or the PLZF may lead to a better understanding of this interaction.

As mentioned in the introduction section, another study found that mGluR5 activation promotes inhibitory phosphorylation of GSK-3β which increases *zbtb16* (PLZF) levels and leads to ubiquitination and proteasomal degradation of the Atg14 protein and inhibition of autophagy (Ibrahim et al., 2021). In addition, a study has demonstrated that a BTB-Kelch protein known as KLHL12 binds to the polymorphic region in the IL3 of the D_4R to function as an adaptor to target the D_4R to an E3 ubiquitin ligase complex (Rondou et al., 2008). Ubiquitination of the membrane proteins has been shown to target them for degradation after endocytosis but can also have other functions, such as internalization, trafficking and signaling (Rondou et al., 2008). Although only a few GPCRs have been described to undergo ubiquitination, such as the β2-AR (Shenoy et al., 2001), the chemokine receptor CXCR4 (Marchese et al., 2003), or the V2

vasopressin receptor (Martin, Lefkowitz, & Shenoy, 2003) for example, it is possible that PLZF as a novel regulator of the D_5R may target the receptor to ubiquitination as a form of degradation. PLZF is similar to KLHL12 as they are both members of the large family of BTB domain-containing proteins, which are adaptors in E3 ligases that are based on the Cul-3 type of Cullin proteins. Furthermore, studies have shown that the Atg14L is controlled by *zbtb16*-Cul-3-Roc1 E3 ubiquitin ligase complex, and there is a wide range of GPCR ligands and agonists that regulate the levels of Atg14L through *zbtb16* (Zhang et al., 2015). Therefore, the PLZF interaction with the D_1R-class could regulate autophagy **(Fig. 32)**.

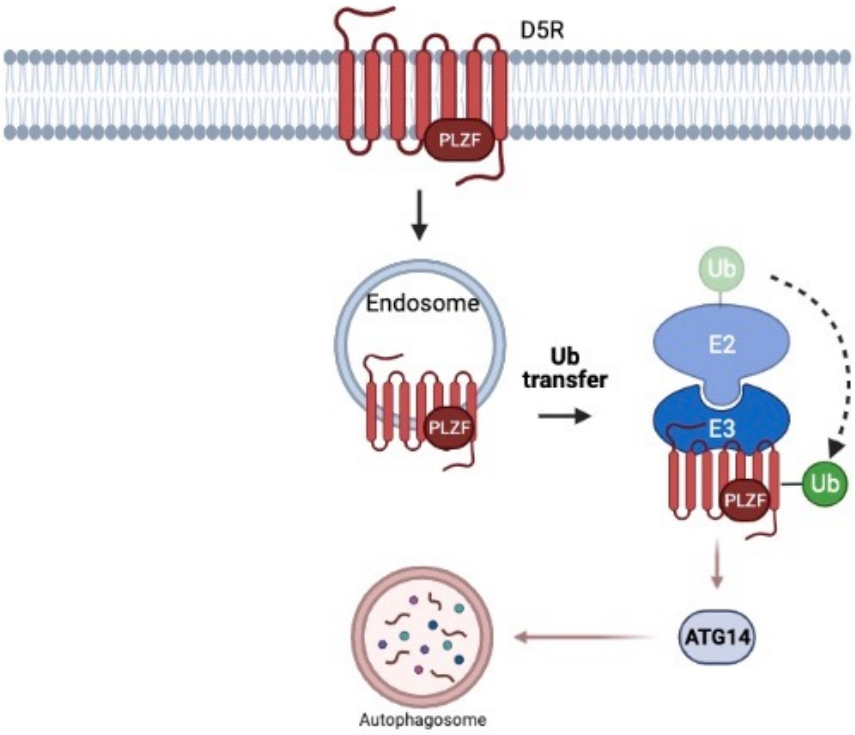


Figure 32. PLZF may promote ubiquitination of the D_5R by targeting the receptor to the Cul-3-based E3 ligase complex
PLZF was identified as a novel interaction partner for the IL3 of D_5R. BTB-domain containing proteins define a class of adaptors in E3 ligases that are based on the Cul-3 type of Cullin proteins. Therefore, PLZF may target the D_5R to the Cul-3-based E3 ligase for subsequent ubiquitination of the D_5R. This interaction may play a role in the regulation of the levels of Atg14 to form autophagosomes and control autophagy.

6. Conclusion

The findings of this MSc. thesis have provided information about the novel role of PLZF in the differential regulation of the D_1R-class in terms of protein interactions, signaling and trafficking mechanisms. The results of this project reveal the similarities and contrasting differences between the D_1R-PLZF and D_5R-PLZF interactions. The results of the co-IP studies demonstrate a significant increase in the association between PLZF and the D_1R-class, upon agonist stimulation. Whereas the co-expression of the β-arrestin 2 with PLZF and the D_1R or D_5R resulted in a decreased recruitment of β-arrestin 2 to the complex, under basal and stimulated conditions. These findings suggest that DA agonist stimulation of the D_1R-class would stabilize the receptors in an active state allowing PLZF to interact further with the receptors. In addition, perhaps the decreased β-arrestin 2 recruitment to the complex suggests that the PLZF protein exhibits an antagonistic effect towards the β-arrestin 2 and results in the distinct signaling and trafficking properties for each receptor subtype.

In the presence of PLZF, there is an observed increase in D_1R-mediated cAMP activation and a decrease in the D_5R-mediated cAMP activation. Interestingly, a similar trend was detected in the D_1R and D_5R-mediated ERK activation, demonstrating a correlative effect between cAMP and ERK. In conformity with these findings, PLZF inhibits the internalization of the D_1R and increases cell surface expression, suggesting that the increase in cAMP activation may be due to increased coupling with the $G\alpha_s$. Moreover, the observed decrease in D_5R-mediated cAMP activation with PLZF may be due to facilitated internalization causing less time for the receptor to be at the cell surface as well as decreased coupling with the $G\alpha_s$. Finally, the PLZF protein also distinctly regulates the recovery of the D_1R in comparison to the D_5R. Since co-IP studies reveal a reduction in β-arrestin 2 recruitment to the D_1R-class in the presence of PLZF, it is

likely that the PLZF co-expression with the D_1R accelerates the recovery of the receptor while delaying the D_5R recovery. Future studies should further explore the D_1R-class and PLZF interaction in vivo, specifically through co-IP in brain tissues such as the cortex, hippocampus and striatum. In addition, the role of β-arrestin 2 in the recovery of the receptors can be further validated through ELISA. This project reveals that the PLZF interaction with the D_1R-class is mutually exclusive and more details about the mechanisms involved in this association may help uncover more details about the distinct regulation of the D_1R and D_5R.

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